Université de Montréal

Influence du système endocrinien de la vitamine D dans la régulation de la vitamine D_3 25-hydroxylase *CYP27A* hépatique et intestinale chez l'humain et le rat

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Influence du système endocrinien de la vitamine D dans la régulation de la vitamine D_3 25-hydroxylase *CYP27A* hépatique et intestinale chez l'humain et le rat

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RÉSUMÉ

La vitamine D_3 25-hydroxylase CYP27A est exprimé principalement dans le foie. Cependant, son expression est également détecté dans les tissus extra-hépatiques tel l'intestin. Le but de notre étude est de vérifier l'effet du statut nutritionnel en vitamine D_3 (D_3) et de la supplémentation en D_3 chez le rat et chez l'humain sur l'expression génique du *CYP27A* hépatique et intestinal, et en corollaire sur l'expression des cytochromes *CYP24* (vitamine D_3 24-hydroxylase) et *CYP27B1* (vitamine D_3 1α-hydroxylase). Nos études chez l'humain ont révélées par analyse de Northern la présence du CYP27A non seulement dans le foie adulte, mais également dans le rein adulte ainsi que le foie et le rein fétal. Les niveaux des ARNm du *CYP27A* sont augmentés dans des specimens d'hépatocarcinome et de métastases intrahépatiques d'origine du colon. Chez le rat, les niveaux des ARNm du *CYP27A* hépatique ne sont pas influencés par le statut nutritionnel en D_3 mais sont regulés par la 1,25 dihydroxyvitamine D_3 ($1,25(OH)_2D_3$) au niveau de la transcription. Par ailleurs, les ARNm du *CYP27A* intestinal sont hautement régulés *in vivo* par tous les métabolites de la D_3, soient la D_3, la 25-dihydroxyvitamin D_3 ($25(OH)D_3$) et la $1,25(OH)_2D_3$ chez le rat. Chez l'humain, les trois hydroxylases ainsi que le récepteur nucléaire de la D_3 (*VDRn*) sont exprimés tout au long de l'intestin et du colon humain fétal. Une comparaison entre les niveaux d'expression des ARNm du *CYP27B1* entre le jejunum et le colon indique une expression génique du *CYP27B1* significativement plus grande dans le colon. Finalement, par culture organo-typique, nous avons observé que la $1,25(OH)_2D_3$ affecte les trois hydroxylases, en abaissant les ARNm des *CYP27A* et *CYP27B1*, et en augmentant les niveaux des *CYP24* et *CYP3A4*. En conclusion, nos études montrent clairement qu'une carence en D_3, l'administration de D_3, de $25(OH)D_3$, de $1,25(OH)_2D_3$, ainsi que certaines pathologies peuvent influencer l'expression du *CYP27A* hépatique et intestinal, à la fois chez l'humain ainsi que chez le rat. Les résultats de ces études représentent un ajout important à la compréhension des effets de la carence en D_3, un phénomène encore largement répandu à travers le monde. De plus, dans l'optique d'une utilisation croissante de certains analogues de la D_3 dans le traitement de différentes pathologies, ces résultats permettront de mieux comprendre les effets de l'administration de ces produits sur l'homéostasie en D_3, ainsi que sur certains autres cytochromes impliquées dans le métabolisme des médicaments.

Mots clés: vitamine D_3, CYP27A (vitamine D_3 25-hydroxylase), CYP24 (vitamine D_3 24-hydroxylase), CYP27B1(vitamine D_3 1α-hydroxylase), CYP3A4 (cytochrome P450 3A4).

SUMMARY

The vitamin D_3 25 hydroxylase CYP27A is predominantly located in the liver. However, its expression is also detected in extrahepatic tissues such as the intestine. The aim of our studies was to investigate the effect of the vitamin D_3 (D_3) status and of D_3 repletion both in the rat (*in vivo*) and in human (*ex vivo*) on *CYP27A* in the liver and the intestine. In parallel, studies with *CYP24*(vitamin D_3 24-hydroxylase) and *CYP27B1* (vitamin D_3 1α-hydroxylase) were also carried out. The human studies reveal through Northern blot analyses the presence of *CYP27A* mRNA not only in adult liver, but also in the adult kidney and in the fetal liver and the kidney. The studies also illustrate that *CYP27A* can be significantly upregulated in certain pathological situations such as hepatic carcinoma and intrahepatic colon metastasis. In the rat, hepatic *CYP27A* mRNA levels are not influenced by the D_3 nutritional status but are transcriptionally regulated by 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Furthermore, intestinal *CYP27A* is highly regulated *in vivo* by all three D_3 metabolites, namely D_3, 25-hydroxyvitamin D_3 ($25(OH)D_3$) and $1,25(OH)_2D_3$. The human fetal intestine expresses all three D_3 hydroxylases, *CYP3A4*, and the nuclear vitamin D_3 receptor (*VDR*) all along the small intestine and colon, with only *CYP27B1* mRNA levels significantly higher in the colon compared to the levels observed in the jejunum. Moreover, when human fetal intestine was harvested and subjected to organotypic culture conditions, all three D_3 hydroxylases and *CYP3A4* were found to be sensitive to $1,25(OH)_2D_3$ administration. Indeed, *CYP27A* and *CYP27B1* mRNA levels were significantly downregulated by the hormone, whereas *CYP24* and *CYP3A4* were upregulated. Our studies indicate that D_3 deficiency, D_3, $25(OH)D_3$, $1,25(OH)_2D_3$ administration as well as malignant transformation can modulate hepatic and intestinal *CYP27A* mRNA expression in the rat and human. These findings are of important relevance for our further understanding the effects of D_3 deficiency, an ever-present widespread problem in today's societies. Furthermore, the increased use of certain D_3 analogues in the treatment of various pathologies will benefit in the better understanding of the effect of D_3 metabolites on the D_3 endocrine system and overall D_3 homeostasis, as well as on other cytochromes such as those involved in drug metabolism.

Key words: vitamin D_3, CYP27A (vitamin D_3 25-hydroxylase), CYP24 (vitamin D_3 24-hydroxylase), CYP27B1(vitamin D_3 1α-hydroxylase), CYP3A4 (cytochrome P450 3A4).

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LIST OF ABBREVIATIONS

vitamin D_3: D_3

$1,25(OH)_2D_3$:1,25-dihydroxyvitamin D_3

$25(OH)D_3$: 25-hydroxyvitamin D_3

$24,25(OH)_2D_3$: 24,25-dihydroxyvitamin D_3

$1,24,25(OH)_3D_3$: 1,24,25-trihydroxyvitamin D_3

CTX: cerebrotendinous xanthomatosis

CYP450: cytochrome P450

CYP3A4: cytochrome P450 3A4

CYP27A: mitochondrial vitamin D_3 25-hydroxylase

CYP27B1: vitamin D_3 1α-hydroxylase

CYP24: vitamin D_3 24-hydroxylase

D-Ca-: vitamin D deficient, hypocalcemic

DBP: vitamin D binding protein

cDNA: complementary deoxyribonucleic acid

FAD: flavin adenosine diphosphate

FMN: flavin mononucleotide

FXR: farnesoid X receptor

NADPH: nicotinamide dinucleotide phosphate, reduced form

PCR: polymerase chain reaction

PKC: protein kinase C

PTH: parathyroid hormone

PXR: pregnane X receptor

mRNA: messenger ribonucleic acid

RT-PCR: reverse transcriptase polymerase chain reaction

RXR: retinoic X receptor

VDR: nuclear vitamin D receptor

VDRE: vitamin D response element

À mes parents,

pour avoir toujours étés là,

pour leur soutien, leur amour inconditionnel, leur inspiration

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CHAPTER 1: GENERAL INTRODUCTION

1.1 CYTOCHROME P450S

The cytochromes P450s (P450s) are monooxygenase enzymes which are members of a superfamily of hemoproteins that play a pivotal role in the metabolism of a wide variety of xenobiotic and endogenous compounds. There is considerable interest in the function of these enzymes due to their involvement in the detoxification of foreign compounds and bioactivation of drugs and carcinogens. Moreover, P450s catalyze key steps in steroidogenesis, as well as in the metabolism of endogenous compounds such as prostaglandins, biogenenic amines, leukotrienes, bile acids as well as cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2). P450s are widely distributed in nature with different isoforms present in plants, insects, some bacteria, yeast and mammals (1).

1.1.1 BIOCHEMICAL PROPERTIES

Reactions catalyzed by the monooxygenase system include hydroxylations, epoxidations, O-, S- and N-dealkylations, and N-oxidations (2). P450s contain approximately 500 amino acids. A cysteine residue molecule located near the carboxyl-terminus of the protein provides the essential thiol-ligand for the heme iron. The amino-terminus of the protein is rich in hydrophobic amino acids and is believed to act as a domain for binding the protein to cell membranes. The P450s isoforms catalyze the nicotinamide dinucleotide phosphate (NADPH) and oxygen dependent oxidative transformation of a large number of different chemical compounds. In general, a specific P450 isoform will catalyze the metabolism of a limited number of chemical structures (such as steroids and fatty acids) while other P450s have a broad substrate specificity suggesting a role for a unique "active site geometry" for some but not all P450s. In tissues such as liver, intestine and the cortex of the adrenal gland, the concentration of P450s greatly exceeds the concentration of other hemeproteins (such as the mitochondrial cytochromes). Indeed, in the rat liver the concentration of P450s can be as high as 50 nmol per gram wet weight of tissue, thereby representing more than one percent of hepatic proteins.

P450s are distributed in almost every organ of the human body, although the type of P450s in a tissue or organ appears to be specific. Moreover, the cellular expression of many P450s is regulated by transcription factors which become activated during exposure to various chemicals.

In general, P450s undergo a cyclic series of reactions (Figure 1.1) where a ferric form of the hemeprotein initially reacts with a molecule to form a complex. This ferric P450-substrate complex is reduced by an electron transported from NADPH. The ferrous substrate complex reacts with molecular oxygen to form a ternary complex of ferrous P450-substrate-oxygen. Carbon monoxide can compete with molecular oxygen resulting in the formation of a carbon monoxide complex of ferrous P450. The carbon monoxide reacts with reduced P450 producing an absorbance band with a maximum at 450 nm in the optical spectrum. The ternary complex of ferrous P450, organic chemical substrate and molecular oxygen is further reduced by a second electron transferred from NADPH. This generates a two electron reduced intermediate (that has not been fully identified) that yields a molecular rearrangement. The chemistry of oxygen incorporation into the substrate results in the complex of ferric P450, which can participate in the metabolism of another substrate.

Central to the operations of the P450s is the need to provide electrons from NADPH (Figure 1.2). Mammalian tissues have two types of electron transport systems operative for different P450s. One type is located in the mitochondria of some cells and consists of a flavin adenosine diphosphate (FAD)-containing reductase and an iron-sulfur protein (called ferredoxin or adrenodoxin). This mini-electron transport system is similar to that found in bacteria where a P450 may be functioning to break down chemicals as an energy source for growth. The second type is associated with the endoplasmic reticulum of many cell types where a unique flavoprotein containing both FAD and FMN (flavin mononucleotide) function as cofactors. The latter type is frequently referred to as the microsomal type of P450 system.

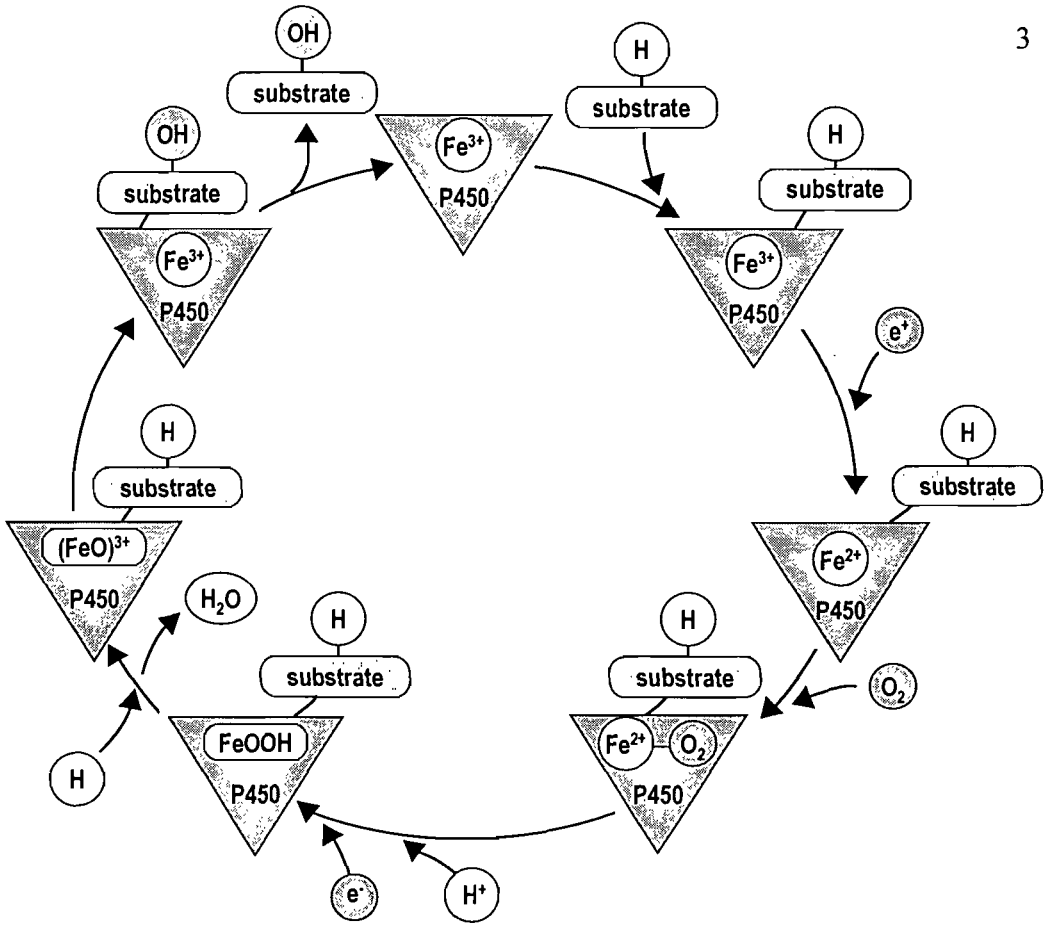

Figure 1.1: Cyclic mechanism for the oxygenation of a substrate by a cytochrome P450 enzyme. After the initial binding of the substrate to the cytochrome P450 (P450) enzyme (upper right), the iron atom (Fe) in the enzyme is reduced (from the ferric [Fe^{3+}] to the ferrous [Fe^{2+}] form) by the addition of an electron (e-) from another donor molecule (a flavin-containing enzyme called NADPH-P450- reductase). The reduction of the iron atom allows it to bind to molecular oxygen (O_2). The addition of another electron and a proton (H^+) to the iron atom forms a FeOOH complex. The loss of a molecule of water produces an $(FeO)^{3+}$ complex, which transfers its oxygen atom to the substrate. The oxidized substrate is released freeing the P450 enzyme to repeat the cycle.

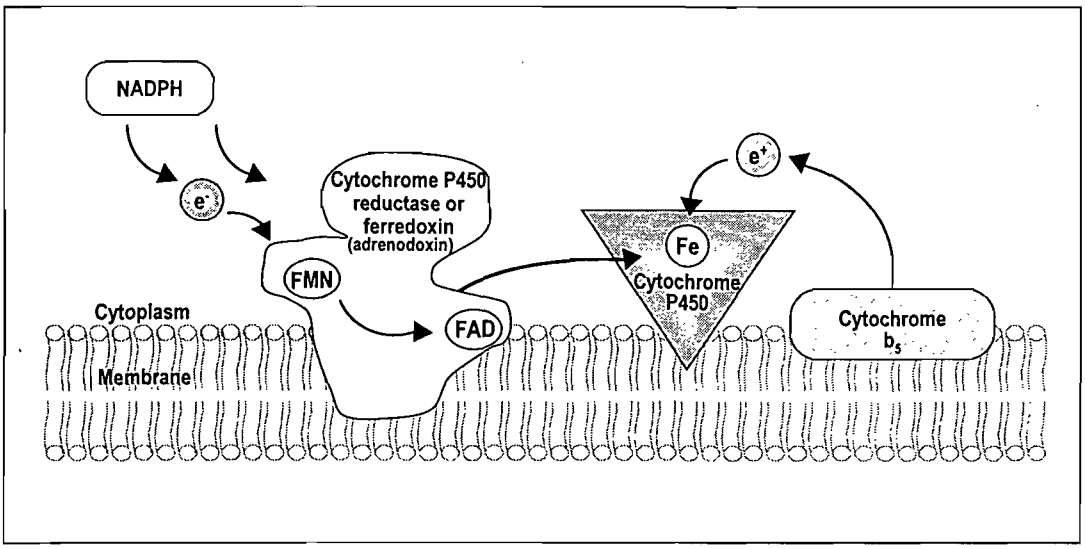

Figure 1.2: Molecular constituents of the cell's cytochrome P450 system lie within the membrane of the endoplasmic reticulum, or the inner mitochondrial membrane. Before the cytochrome P450 (P450) enzyme can oxidize a substrate, the iron atom in the enzyme's heme group must accept two electrons from either one of two molecules: NADPH, located in the cytoplasm, or cytochrome b_5, which is embedded in the membrane. P450 reductase, which contains two subcomponents, FAD and FMN, acts as an intermediary molecule in the transfer of electrons from NADPH. The FAD containing reductase equivalent in mitochondria is termed ferredoxin (or adrenodoxin).

1.1.2 CLASSIFICATION

P450 enzymes are ubiquitous in living organisms, and more than 400 individual isoforms have been thus far identified and sequenced from plants, animals, bacteria and yeast (3). Based on their sequence, P450s are classified into gene families and subfamilies. Members of the same family exhibit at least 40% amino acid sequence identity, while within a subfamily the identity is greater or equal to 55% (3).

Furthermore, based on their requirements for the redox partner, P450 enzymes can be divided into 2 main groups: class I enzymes, found in mitochondria and class II enzymes, more abundant in endoplasmic reticulum. Class I enzymes require both an iron-sulfur protein (ferredoxin) and an FAD-containing NAD(P)H-ferredoxin reductase for catalysis, whereas class II P450s require an FAD/FMN-containing NADPH-P450 reductase, although they may utilize an additional electron donor, namely cytochrome b_5. Finally, there are class III enzymes that do not require any exogenous source of electrons, the latter being obtained from a peroxide substrate (i.e. prostacyclene synthase). In mammals, the main xenobiotic metabolizing enzymes belong to class II and comprise families 1-4 (3). In contrast, P450s involved in steroidogenesis are split between class I and class II, with for example CYP7α, CYP11 and CYP27A being class I families and CYP17, 19 and 21 being class II families. Over half the families (nine) of human P450s are associated with cholesterol and steroid hormone metabolism. Four families of P450s are located within mitochondria and therefore use the mini-electron transport chain containing an iron sulfur protein. As mentioned above, many forms of P450s display broad substrate specificities, yet they often exhibit strict regio- and stereospecificity toward a particular compound.

1.1.3 INHIBITIONTION AND INDUCTION OF P450 CYTOCHROMES

Impairment of any of the steps of the P450 catalytic cycle can lead to inhibition. The binding of the substrate to the ferric form of the enzyme, binding of molecular oxygen and substrate oxygenation are particularly vulnerable to inhibition (4). The mechanisms of P450 inhibition can be grossly divided into 3 categories: reversible inhibition, quasi-irreversible inhibition and irreversible inhibition (5). Among these, reversible inhibition is probably the

most common mechanism responsible for documented drug interactions. Ketoconazole, a broad spectrum imidazole antimycotic agent, is an example of a non-specific competitive inhibitor of P450-catalysed reactions. In mechanistic terms, reversible interactions arise as a result of competition at the P450 active site and probably involve only the first step of the P450 catalytic cycle. On the other hand, agents that act during or subsequently to the oxygen transfer step are generally irreversible or quasi-irreversible inhibitors. Both irreversible and quasi-irreversible inhibition are caused by the formation of reactive metabolites. Thus, the irreversible and quasi-irreversible inhibition require at least one cycle of the catalytic process.

One of the intriguing aspects of the P450s is that only some of these enzymes are inducible. Unlike inhibition, which is an almost immediate response, induction is a slow regulatory process that can reduce drug concentrations in plasma, and may compromise the efficacy of the drug in a time-dependent manner. From a biological point of view, induction is an adaptive response that protects the cells from toxic xenobiotics by increasing the detoxification activity.

Induction of P450 enzymes occurs predominantly at the level of transcription (6-8). A notable exception is the well-studied alcohol inducible CYP2E1 gene, the induction of which involves a post-transcriptional mechanism (7;9). Induction generally occurs at sites of exposure or excretion of xenobiotics, such as in the liver, lung, skin and other tissues. Depending on the chemical nature of the xenobiotic, P450 isozymes belonging to a particular sub-family are predominantly induced. For example, the major isozymes induced by polycyclic aromatic hydrocarbon, such as coal tar constituents (benzopyrenes and anthrenes) and by-products of paper bleaching (dibenzodioxins) are those of the CYP1A sub-family (10). In contrast, chlorinated hydrocarbon pesticides (such as DDT, chlordane, dieldrin) and therapeutics (such as the sedative phenobarbital) predominantly induce isozymes of the CYP2B sub-family (11). Members of the CYP4A sub-family are chiefly induced by the plasticizing agent 2-ethylhexyl phthalate and by other peroxisomal proliferators, such as the antilipidaemic drug clofibrate (12;13). The glucocorticoid dexamethasone (7) and metyrapone (14) predominantly induce isozymes of the CYP3A sub-family. It should be noted that there are often species-specific P450 isozymes and that some P450 isozymes can be differentially induced in different species. The molecular mechanisms by which various hydrophobic foreign chemicals increase the transcriptional activation of species specific

P450 genes constitute a fascinating problem of gene regulation. Most of the information on P450 gene regulation has been obtained from animal studies. Important interspecies differences also exist in the response of other inducible subfamilies of P450s. Specifically, phenobarbital induces predominantly members of the CYP2B subfamily in rats, whereas in humans it appears that the major form induced belongs to the CYP3A subfamily (15). Furthermore, members of the CYP3A subfamily in rats are inducible by the steroidal agent, pregnenolone-16α-carbonitrile, but not by the antibiotic rifampicin. The opposite is true in rabbits and humans (16). Thus, drugs that induce P450 enzymes in animals should not be assumed necessarily to have enzyme-inducing capacity in humans and vice-versa.

1.1.4 Cytochrome P450s in Vitamin D_3 metabolism

Vitamin D_3 (D_3) is a natural secosteroid that has, in its native form, virtually no biological activity. Once in circulation, D_3 is taken up by the liver and hydroxylated at C-25 by a P450-dependent mixed function oxidase systems (Figure 1.3). At physiological concentrations, 25-hydroxyvitamin D_3 ($25(OH)D_3$) has no biological activity and must be hydroxylated at position C-1α (principally in the kidney) into 1α,25 dihydroxyvitamin D_3 (calcitriol, $1,25(OH)_2D_3$) in order for the vitamin to achieve hormonal status and thus its full biological potentialThere are four P450s required for the activation and inactivation of D_3 (cholecalciferol). 7-dehydrocholesterol, a late intermediate in the cholesterol biosynthetic pathway is photolyzed by UV light producing D_3. The vitamin is first hydroxylated at position C-25 by a mitochondrial and/or microsomal enzyme. The mitochondrial D_3 25-hydroxylase, CYP27A, has been identified, whereas the status and role of the D_3 microsomal entity remains questionable. The product of this reaction, $25(OH)D_3$ is then converted to $1\alpha,25(OH)_2D_3$ by a mitochondrial P450, the vitamin D_3 1α-hydroxylase, CYP27B1. This enzyme has been known to exist in kidney mitochondria for decades but because of its very low level of expression and presumed instability, it had been virtually impossible to purify. Only within the past four years has it been cloned and found to be a member of the CYP27A family (17-20). The C-1α hydroxylation generates the active and hormonal form of D_3, $1\alpha,25(OH)_2D_3$. A third mitochondrial P450, vitamin D_3-24 hydroxylase (CYP24), inactivates calcitriol by hydroxylation on the side chain at position C-24 which greatly

reduces binding to the nuclear vitamin D_3 receptor (VDR).

VITAMIN D_3 METABOLISM

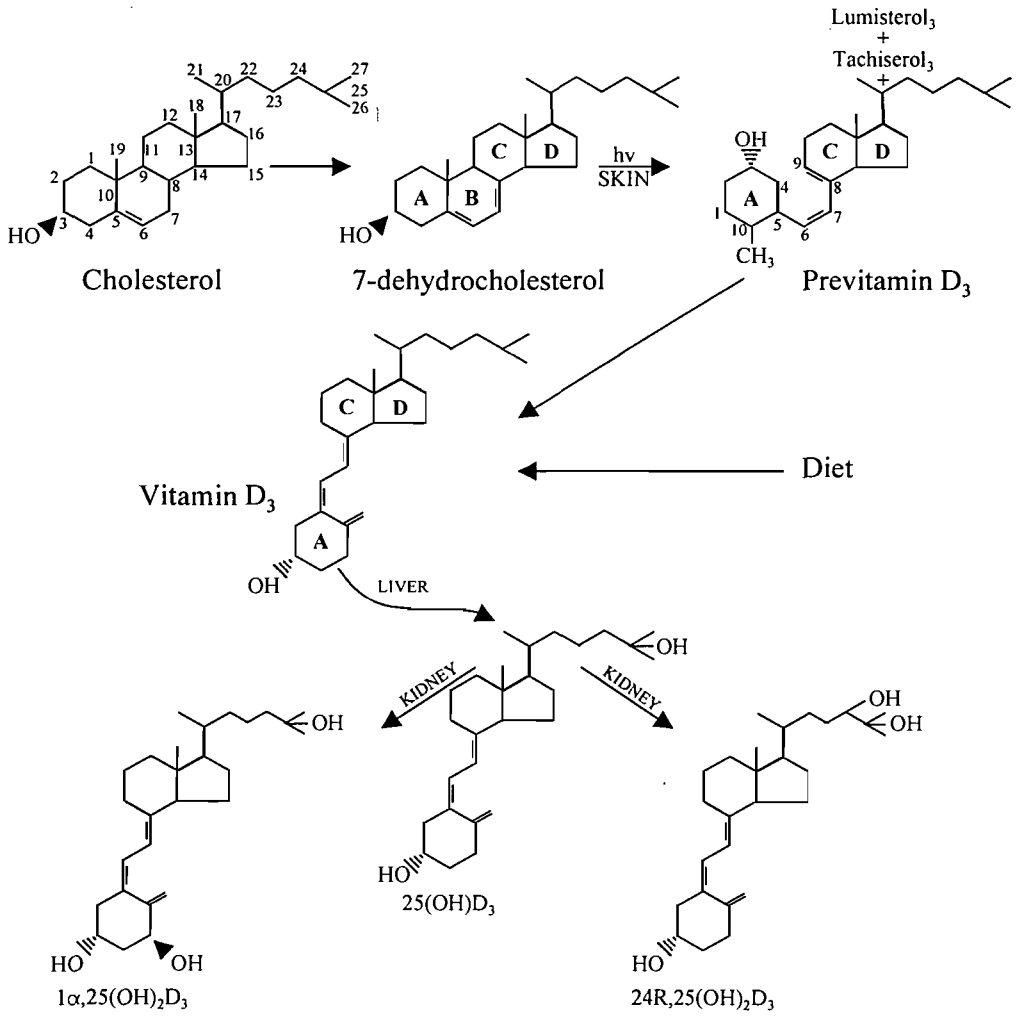

Figure 1.3: Key vitamin D sterol structures. Top row: vitamin D_3 (D_3) is structurally related to cholesterol (the numbering of the carbon atoms is identical). D_3 is produced (in the skin) from 7-dehydrocholeserol by a UV-mediated photochemical reaction. The immediate photochemical product, preD_3 then thermally equilibrates (over time) into D_3. Middle row: conformational representation of D_3. Structure presented is an extended version derived from X-ray crystallographic analysis; Bottom row: structures of the three principal metabolites of D_3.

1.2 VITAMIN D METABOLISM

1.2.1 VITAMIN D_3 25-HYDROXYLASES

1.2.1.1. Evidence for microsomal and mitochondrial D_3 25-hydroxylase activity

As previously mentioned 25-hydroxylation of D_3 is catalyzed by P450 dependent enzyme systems in both mitochondrial (21;22) and microsomal fractions (23;24). The relative importance of the two 25-hydroxylase systems *in vivo* is not known, but it is generally believed that the microsomal 25-hydroxylation of D_3 is more important than the mitochondrial 25-hydroxylation in the bioactivation process under normal conditions (25;26). In humans, the mitochondrial D_3 25-hydroxylase was believed to be the predominant and only form expressed (27), although a microsomal form is also active (28).

There is abundant evidence that the 25-hydroxyvitamin D_3 1α-hydroxylase, as well as the degradation system 25-hydroxyvitamin D_3 24-hydroxylase, are highly regulated (29-31). However the question of whether the 25-hydroxylase is regulated remains undetermined.

Interestingly, the D_3 25-hydroxylase that has been described to date, in terms of gene regulation, is the enzyme CYP27A of mitochondrial origin. The porcine microsomal D_3 25-hydroxylase has been cloned (32), although its presence remains to be characterized in other species (33). This microsomal 25-hydroxylase identified can catalyse 25-hydroxylation of both D_3 and D_2(34). A cDNA encoding the pig liver microsomal D_3 25-hydroxylase, as deduced by both DNA sequence analysis, showed 70-80% identity with members of the CYP2D subfamily and has been assigned the name CYP2D25 (35). However, the only CYP2D enzyme known to be expressed in man is CYP2D6. This enzyme is polymorphically expressed and is lacking in 5-10% of the caucasian population. It is not known whether D_3 25-hydroxylation in man could be catalysed by CYP2D6.

1.2.1.2 Biochemical and Molecular aspects of mitochondrial and microsomal D metabolism

The microsomal 25-hydroxylase fraction of rat liver and was influenced by the D status (36-38) and required the presence of a cytosolic factor for optimum activity. 25-

hydroxylation of the synthetic compound dihydrotachysterol (DHT3)was not regulated compared to that of the natural substrate D_3(38). The presence of the enzyme was confirmed to be a rat liver microsomal P-450 (24;39), requiring a soluble cytosolic factor (for full reconstitution of enzyme activity) (39). In 1983, a rat microsomal cytochrome P450 active in C-25 hydroxylation of bile acid intermediates 5β-cholestane-3α,7α,12α-triol, cholestane-3α,7α-diol as well as D_3 and 1α (OH)D_3 but not D_2 (40). A purified and partially sequenced P450 active in 25-hydrxylation of D_3 with an inhibitor removed from the microsomes during the purification steps (41), and did not require the presence of a cytoplasmic factor.

In 1980, Bjorkhem and Holmberg studied the activities of a P450 preparation solubilized from rat liver mitochondria obtained from rats which had been treated with phenobarbital or subjected to a rachitogenic diet (22). They observed that both phenobarbital and the rachitogenic diet increased C-25 hydroxylation of D_3 but had little effect on the C-27 hydroxylation of the C-triol. These observations led the investigators to conclude that 2 distinct P450 species existed, each responsible for the hydroxylation of cholestanetriol and D_3. At the same time, Masumoto et al purified to homogeneity the D_3 25-hydroxylase from rat liver mitochondria and characterised it as a P450 catalysing the C-25 hydroxylation of both D_3 and 1α (OH)D_3 (42). These researchers proposed that a single enzyme was involved in the C-27 hydroxylation of 5β-cholestane-3α,7α,12α-triol and in the C-25 hydroxylation of D_3, although the preparation exhibited much lower activity toward D_3 compounds than toward the C-27 hydroxylation of bile acid intermediates. D_3 competitively inhibited the 27-hydroxylation of C-triol, whereas C-triol inhibited D_3 25-hydroxylation (42), concluding that both substrates were catalysed at a common active site on a single protein. D_3 25-hydroxylation and C27 sterol hydroxylation activities were located , probably exclusively, in the inner mitochondrial membrane matrix (27;43).

Heterologous cell systems confirmed the identity of sterol 27-hydroxylase and D_3 25-hydroxylase as well as the requirement for adrenodoxin and adrenodoxin reductase but not NADPH P450 reductase (44).

1.2.1.3. CYP27A (MITOCHONDRIAL VITAMIN D_3 25-HYDROYLASE)

1.2.1.3.1 The CYP27A gene

CYP27A, the mitochondrial D_3 25-hydroxylase, has been cloned in several species including the rabbit (44), rat (45;46), and human (47;48). The gene has been shown to be located on chromosome 2q33-qter (49). Northern analyses demonstrate two mRNA sizes of 1.9 and 2.3kb for *CYP27A* in liver and fibroblasts (46;48). Characterization of the 2.3kb mRNA indicated a sequence identical to the 1.9kb mRNA in its protein-coding region, except for ~ 400-nucleotides in an extended sequence at its 5' end (50). The rat *CYP27A* gene contains 11 exons of 80-415 nucleotides that are separated by 10 introns of 83 bases to ~ 10kb (51). The protein sequence of the human enzyme has been reported to be 72% identical to the rat and 81% identical to the rabbit *CYP27A* (45;48). The mature protein contains 444 to 501 amino acids depending on the species involved and the rat enzyme molecular weight has been estimated to be 51kDa (45).

1.2.1.3.2 CYP27A promoter and regulation studies

Functional assays showed that the –217/-10 nucleotide region for the translation start site (minimal promoter), devoid of TATA and CAAT boxes, contains all the elements for basal transcription (52). Possible positive transcription regulation sites are located at position –187 to –320 and –857 to 1087bp (53). A negative transcription regulator site is located in position –320 to –413bp. An enhancer sequence is located upstream to position –1087 (53). A putative bile acid response element (BARE) is also located between –110 and –86bp of the CYP27 promoter , on which HNF1α and C/EBP bound (54).

Footprinting analysis of the minimal promoter showed four protected region. Three of the regions contained and Sp1 binding site, and one an HNF4 site. Electrophoretic mobility shift assays demonstrated that SP1, Sp3, and HNF4 transcription factors bind these sites (52). Mutagenesis of any of these sites resulted in the loss of promoter activity. Sp1, Sp3 and HNF4 were found to cooperate in the expression of the human CYP27A gene in HepG2 cells (52). A dexamethasone responsive element was found to be located between 1087 and 678bp upstream to the putative ATG. The cyclosporin A-responsive element is mapped to between 1087 and 4000 bp upstream of the ATG (53). Cholic acid represses sterol 27-hydroxylase mRNA level by affecting the stability of its mRNA (53).

1.2.1.3.3. CYP27A regulation not related to vitamin D metabolism

CYP27A is a high-capacity enzyme involved primarily in the hydroxylation of bile acid intermediates, but its substrate specificity is broad and exceeds the field of bile acid biosynthesis. The purified enzyme has been shown to be active on both the C-25 and C-27 hydroxylation of cholesterol as well as on other bile acid intermediates (55;56). Rat hepatic CYP27A is highly sensitive to the prevailing concentrations of bile acids with increases in enzyme activity, steady state mRNA level and the rate of gene transcription being observed after interruption of the enterohepatic circulation (57;58). Physiological concentrations of insulin also down-regulate *CYP27A* gene transcription through a direct effect of the hormone on the transcription rate in hepatocytes (59).

CYP27A is also sensitive to pituitary-regulated steroids, growth hormone and the diurnal rhythm, with a two fold increase in enzyme activity observed in the mid-dark compared to the mid-light period (57). Both the 1.9 and 2.3kb mRNA species appear to be modulated by the physiological state of the animal and regulated by growth hormone in parallel to the serine protease inhibitor mRNAs (50).

1.2.1.3.4. CYP27A regulation related to vitamin D metabolism

Most regulation studies on *CYP27A* have been linked to the metabolism of cholesterol and bile acids. Few studies, until recently, have addressed the effect of these compounds on the handling of D_3.CYP27A also catalyzes the C-24, 25 and 27 hydroxylation of D_3, D_2 and related compounds (47). While D_3 substrates were found to be preferably hydroxylated at C-25, Guo *et al.*, (47) observed that when the substrates exhibited the ergocalciferol side chain, such as that found in D_2 or $1\alpha(OH)D_2$, a predominance of 24-hydroxy metabolites occurred (metabolites which have also been found *in vivo* in rat, cow and chicken) (60-62). In addition, some production of C-27 hydroxylated products also occured (63). Surprisingly, D_2 was not, while $1\alpha(OH)D_2$ was only poorly hydroxylated at position C-25 (47).

In addition, several laboratories have now shown that the enzyme prefers 1α-hydroxylated analogs of D_3 or D_2 over their non-hydroxylated counterparts. This includes

the natural substrate D_3, which is hydroxylated at C-25 several times less efficiently than $1\alpha(OH)D_3$ (22;35;47).

Of the studies examining regulation of CYP27A by the D_3 status, Axén *et al.* (64) reported that renal and hepatic *CYP27A* was affected by $1,25(OH)_2$ D_3 administration but that kidney *CYP27A* mRNA was decreased to a larger extent than that of the liver. Furthermore, *CYP27A* mRNA has been shown to be inducible by D_3 in keratinocytes (65).

1.2.1.3.5 Vitamin D and mineral metabolism in CTX humans and in the CYP27-/- mouse

Mutations affecting the expression of *CYP27A* or its primary sequence leads to cerebrotendinous xanthomatosis (CTX) (66;67), which is an inherited disorder of sterol metabolism and storage characterized by atherosclerosis and progressive neurological dysfunction. CTX is a rare autosomal recessive neurometabolic disease involving lipid metabolism. The classical phenotype, due to mutations in the CYP27 gene, is characterized by neurologic dysfunction, tendon xanthomas and juvenile cataracts.

Bone fractures occurred frequently in patients with CTX (68). Serum 25(OH)D levels were in the low to normal range whereas $24,25(OH)_2D$ levels were markedly decreased. Serum concentration of $1,25(OH)_2D$, Ca, inorganic phosphorus, alkaline phosphatase, parathyroid hormone and calcitonin were normal (69).Interestingly, both normal (70) and abnormal (67;69;71) D_3 and/or calcium metabolism have been reported which suggests the presence of more than one enzyme active in the 25-hydroxylation of D_3. However, some CTX patients have been shown to retain residual C-27 hydroxylase activity (72). The creation of a *CYP27A-/-* mouse has not provided any further insight into the role of CYP27A in relation to D_3 homeostasis. Indeed, the *CYP27A-/-* mouse surprinsingly displays elevated levels of 25(OH)D, and no CTX abnormalities (73). The latter observations suggest alternative C-25 hydroxylation pathway(s) in the *CYP27A* ablated mouse possibly through the microsomal D_3 25-hydroxylase.

The circulating concentrations of 25(OH)D was however somewhat higher in the CYP27-/- than in the CYP27 +/+ mice whereas the corresponding concentrations of $1,25(OH)_2D$ were similar in the two groups (73). Thus it seems less likely that sterol 27-hydroxylase is of importance in formation of 25(OH)D and $1,25(OH)_2D$ in this species.

1.2.2 VITAMIN D_3 24-HYDROXYLASE (CYP24)

1.2.2.1 The vitamin D_3 24-hydroxylase gene

The purification of the rat kidney mitochondrial D_3 24-hydroxylase enzyme and the raising of a rabbit polyclonal antibody (74;75) were milestone achievements that led to the isolation of a cDNA clone for the rat 24-hydroxylase by immunological screening (76). The mRNA contains an open reading frame of 514 amino acid residues that includes the mitochondrial signal sequence. Full-length cDNA clones were subsequently isolated for the human (77) and mouse (78) D_3 24-hydroxylase proteins. The human D_3 24-hydroxylase showed about 80% amino acid sequence identity with both the rat and mouse homologs, whereas the latter two where 95% identical. D_3 24-hydroxylase was not more than 30% identical with any other P450s sequence reported to date. The highest homology (30% identity) was found to be with *CYP27A* (45).

Genomic clones for rat (79) and human (77) D_3 24-hydroxylase have been isolated. The rat gene is a single copy gene that spans about 15kb and contains 12 exons (79). The intron-exon arrangement of the gene most closely resembles that of the CYP22 family (79). The transcription start site for the rat *CYP24* and a likely TATA box have been located and several possible control elements have been identified in the promoter including vitamin D response elements (VDREs), CCAAT, GC, and TATA binding sites (79;80). Similar binding sites for the transcription factors are also present in the promoter for human *CYP24* (81).

Although three VDREs have been identified in the rat CYP24 promoter (82), only two functional elements have been identified on the antisense strand (30;80;83). Initial studies established the binding of VDR as an retinoid-X-receptor (RXR) complex at VDRE-1, which was verified and extended to include VDRE-2 through supershift analysis with a specific monoclonal RXR antibody (82). When analyzed separately, VDRE-2 showed about 4- to 5- fold higher affinity for the VDR-RXR complex than VDRE-1. There is transcriptional synergism between the VDREs, as the wild type induction (18 fold) is greater than the sum of the individual contributions of VDRE-1 (6 fold) and VDRE-2 (3 fold). Two VDREs for

the human promoter have been identified by Chen and DeLuca (81).

1.2.2.2 Catabolism of 1,25 dihydroxyvitamin D_3

CYP24 directs the synthesis of $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$ (84). The two D_3 metabolites express less biological activity than $1,25(OH)_2D_3$, although $24,25(OH)_2D_3$ is suggested to have focused actions in bone and cartilage. Both metabolites also represent initial reactants in the C-24 oxidation pathway that leads to metabolite inactivation via generation of 23- or 24-COOH end products (85;86). Acquisition of a C-24 hydroxyl group is required for entry into the oxidation pathway, which is followed by oxidation of the 24-hydroxyl group to a keto (oxo) function. The 23- and 24-hydroxylase activities are usually coexpressed in the same target cells, with the 24-hydroxylation constituting the major activity (87).

1.2.2.3 Cellular expression

The D_3 24-hydroxylase enzyme displays a broad tissue distribution. A major route of enzyme induction involves the combined action of $1,25(OH)_2D_3$ and of VDR to increase transcription of the *CYP24* gene. Consequently, cellular expression of CYP24 is linked tightly to the coexpression of VDR. Most cells that contain VDR, therefore, express a basal level of CYP24 or respond to increased $1,25(OH)_2D_3$ levels by inducing the biosynthesis of the enzyme, particularly in kidney and small intestine (88;89). Basal expression under normal calcium-homeostatic conditions has been documented for kidney, intestine, bone, placenta, skin (keratinocytes), and macrophages (88-90).

Particular attention has been given to expression of the enzyme in the kidney, which is one of the major sites of CYP24 activity. As this tissue is the major site of $1,25(OH)_2D_3$ synthesis, it seems likely that the high basal *CYP24* expression in kidney represents induction by endogenous $1,25(OH)_2D_3$. In the kidney, enzyme expression has been localized to the proximal tubule and does not display the more general distribution as noted for VDR (91). In parallel, *CYP24* expression in bone occurs in osteoblasts and may play an important role in bone mineral dynamics. The placenta expresses 24-hydroxylase activity and

developmental expression of *CYP24* is controlled by $1,25(OH)_2D_3$-dependent transcriptional induction (92). In addition, investigations with cultured cells have revealed induction of the *CYP24* gene in many different cell types (93-95).

This widespread distribution of the D_3 24-hydroxylase supports a major role for the enzyme in regulating the local concentration and action of the hormone $1,25(OH)_2D_3$.

1.2.2.4 Regulation of expression

Consistent with the general tissue distribution of CYP24, a broad spectrum of regulatory agents act to control cellular expression of the enzyme. Steroid and peptide hormones function through the signal transduction and transcription pathways to alter CYP24 activity. The enzyme level is also changed in response to age and several genetic mineral metabolism disorders.

The major regulators of the D_3 24-hydroxylase are PTH and $1,25(OH)_2D_3$. *In vivo* administration of PTH to thyroparathyroidectomized rats decreases renal production of $24,25(OH)_2D_3$, whereas administration of $1,25(OH)_2D_3$ increases $24,25(OH)_2D_3$ production. The mechanisms of action of PTH and $1,25(OH)_2D_3$ on D_3 24-hydroxylase appear to be similar to the actions of the respective hormones on 1α-hydroxylase in that PTH acts by a mechanism involving cAMP and the $1,25(OH)_2D_3$ action requires new protein synthesis.

Administration of a single dose of $1,25(OH)_2D_3$ to rats markedly increases *CYP24* mRNA level in the kidney and intestine (96;97). In contrast, *CYP24* mRNA is not induced by $1,25(OH)_2D_3$ in hepatocytes, which do not express any 24-hydroxylase activity. Induction of *CYP24* mRNA by $1,25(OH)_2D_3$ has been further studied in primary cultures of rat tubular cells and human colon cancer cells (98;99). This induction is inhibited by the addition of actinomycin D, 5,6-dichloro-1-α-D-ribofuranosyl benzimidazole, and cycloheximide, indicating that the *de novo* syntheses of *CYP24* mRNA and protein are required (98). It has also been reported that phorbol 12-myristate 13-acetate (TPA) alone has no effect, but TPA produces a marked increase in the expression of *CYP24* mRNA in the presence of $1,25(OH)_2D_3$ (98). TPA also shifted the stimulatory dose-response curve of $1,25(OH)_2D_3$ to the left. It is, therefore, hypothesized that the $1,25(OH)_2D_3$ effect leading to *CYP24* induction may occur at a physiological concentration of the hormone by a mechanism

involving protein kinase C (PKC).

1.2.3 VITAMIN D_3 1α-HYDROXYLASE (CYP27B1)

1.2.3.1 THE VITAMIN D_3 1α -HYDROXYLASE GENE

Until recently, analysis of the expression and function of the D_3 1α-hydroxylase in the kidney has been dependent on relatively insensitive enzyme assays using tissue homogenates, mitochondrial enriched fractions, or single nephron sections obtained from D-deficient animals (100;101). Further studies also identified the D_3 1α-hydroxylase in the distal tubule and collecting ducts.

In 1997, Takeyama *et al.*(17), using a VDR knockout model, isolated a candidate 1α-hydroxylase 2.5kb cDNA corresponding to a 507 amino acid P450-like protein, with a predicted size of 55kDa. The overall sequence identity of this CYP27B1 to related mitochondrial cytochrome P450 proteins is limited, ranging from 39% (CYP27A) to 33% (11β-hydroxylase) (20). *CYP27B1* gene spans approximately 6kb, consists of 9 exons and has approximately 500bp of 5' untranslated mRNA.

In parallel with the original cloning of the human gene (20), St-Arnaud *et al.*, (102) isolated the cDNA for the rat *CYP27B1*, which was found to have 82.5% identity to human cDNA. The latter report also confirmed the location of the human *CYP27B1* gene on chromosome 12q13-1-q13-3. This provides further evidence that abnormal *CYP27B1* gene expression is the cause of hereditary pseudovitamin D-deficiency rickets, a disease reported to reside on the above mentioned chromosome.

1.2.3.2 Renal distribution

Data using normal human kidneys confirmed the expression of mRNA and protein for the D_3 1α-hydroxylase in proximal tubules (103). However protein and mRNA were also expressed in distal tubules and in collecting ducts (103). The specificity of the D_3 1α-hydroxylase expression in the kidney was emphasized by stringent controls for both *in situ*

hybridization and immunohistochemistry analyses. The other key sites of D_3 1α-hydroxylase expression along the nephron were the medullary collecting ducts and the papillary epithelium (104).

Although previous studies of the renal function of D_3 have focussed primarily on the production and function of $1,25(OH)_2D_3$ in proximal tubules, there is increasing evidence of a role for the hormone in more distal parts of the nephron. In view of studies with D_3 deficient animals, it is speculated that production of $1,25(OH)_2D_3$ in the proximal tubules acts in an endocrine fashion to support circulating levels of $1,25(OH)_2D_3$, whereas in more distal areas of the nephron it may fulfill an autocrine or paracrine function. Indeed, previous studies have shown that $1,25(OH)_2D_3$, as well as calcitonin and PTH, stimulate calcium reabsorption in the distal nephron (105-108). It is also important to recognize that in some cases, the impact of $1,25(OH)_2D_3$ on renal function may occur through indirect mechanisms. In particular, the observation that the calcium-sensing receptor is primarily regulated by $1,25(OH)_2D_3$, and not by PTH or calcium suggests that this may be the key target for local production and action of $1,25(OH)_2D_3$ in the distal nephron (109).

1.2.3.3 Extra-renal expression

The original description of extra-renal D_3 1α-hydroxylase expression was based on studies of the granulomatous disease sarcoidosis, which is frequently associated with hypercalcaemia (110;111). Enzyme activity analyses using lymph node homogenates and pulmonary alveolar macrophages from patients with sarcoidosis showed high levels of D_3 1α-hydroxylase activity (112-114). Furthermore, addition of exogenous $1,25(OH)_2D_3$ did not appear to inhibit macrophage D_3 1α-hydroxylase as is classically observed with its renal counterpart. This would explain the apparently unregulated synthesis of $1,25(OH)_2D_3$ which is characteristic of the more severe forms of this disease. However, these data also suggested that the expression and regulation of D_3 1α-hydroxylase in extra-renal tissues was different from that observed with the kidney enzyme. It now appears that renal and extrarenal $D_3$1α-hydroxylase activity is due to a single gene product. Therefore, the most likely explanation is that induction of extra-renal D_3 1α-hydroxylase involves regulatory pathways that differ

from renal, cAMP-mediated mechanisms, less sensitive to autoregulation by $1,25(OH)_2D_3$. Induction of extra-renal D_3 1α-hydroxylase frequently involves antigenic mediated activators such as lipopolysaccharide or inflammatory mediators such as interferon-γ. Since these agents use nuclear factor NFκB as signaling mechanism, it can be postulated that this pathway activates D_3 1α-hydroxylase in a manner unlike that of calciotrophic factors and, as a consequence show a differential sensitivity to feedback control by $1,25(OH)_2D_3$. Further analysis of signal-transduction pathways involved in regulating D_3 1α-hydroxylase will be crucial to the understanding of the way in which $1,25(OH)_2D_3$ functions in extra-renal tissues. Using immunohistochemistry and Western analyses with renal D_3 1α-hydroxylase antisera, the enzyme was detectable in tissues such as normal skin (stratum basalis) and sarcoid lymph nodes (115). The D_3 1α-hydroxylase was also highly expressed in skin from sarcoid patients. Immunohistochemistry also confirmed previous enzyme activity studies which indicated that D_3 1α-hydroxylase was expressed in decidual cells (116;117). However, the enzyme was also detectable in trophoblasts and syncytiotrophoblasts, suggesting potentially diverse functions for the hormone in placenta and feto-placental physiology (118). Novel sites for D_3 1α-hydroxylase expression include the parathyroids, pancreas, adrenal medulla, colon and cerebellum, while negative tissues include the heart, liver (hepatocytes) and adrenal cortex.

1.2.3.4 Regulation of vitamin D_3 metabolism

The apparent widespread distribution of protein and mRNA for the D_3 1α-hydroxylase in both renal and extra-renal tissues has raised important questions concerning the local enzyme activity at these sites. The relationship between expression of the CYP27B1 and actual synthesis of $1,25(OH)_2D_3$ in a particular tissue probably involves two specific mechanisms, the first of these being substrate access, and the second being auto regulation of D_3 1α-hydroxylase activity by $1,25(OH)_2D_3$ itself. The former questions the assumption that, in common with other steroid hormones, $1,25(OH)_2D_3$ enters cells by a passive mechanism by virtue of its lipophilic nature. Circulating vitamin D (D) metabolites can bind to a variety of serum proteins, but by far the most important of these is the vitamin D binding

protein (DBP), which is synthesized in the liver. DBP may play an active role in directing D responses (119) because of its relatively high capacity for binding $25(OH)D_3$, making it a likely key determinant of the availability of the substrate to CYP27B1. Recent studies have shown that DBP and DBP-bound D metabolites are filtered through the glomerulus and reabsorbed by the luminal endocytic receptor megalin (gp330) in the proximal tubules (120). Megalin is also expressed in a variety of tissues (121). Thus megalin-mediated endocytosis of DBP-bound $25(OH)D_3$ may act as an additional mechanism controlling tissue-specific synthesis of $1,25(OH)_2D_3$ by modulating the availability of substrate to the CYP27B1 protein. Recently, cubulin, a membrane-associated protein colocalizing with megalin, has been identified. It facilitates the endocytic process by sequestering steroid-carrier complexes on the cellular surface before megalin-mediated internalization of the cubulin-bound ligand (122). Interestingly, dogs with an inherited disorder affecting cubulin biosynthesis exhibit abnormal D metabolism (122).

During D sufficiency, $1,25(OH)_2D_3$ production by the kidney is very tightly regulated. On the other hand, there is a striking up-regulation of the D_3 1α-hydroxylase activity in proximal tubular cells in D-deficient states (123;124). This response appears to be a function of several direct and indirect mechanisms, including changes in accessory proteins such as ferrodoxin, or alterations in VDR or 24-hydroxylase expression. Studies *in vivo* suggest that the key activator of CYP27B1 is PTH and that this effect is mediated, at least in part, by target-cell induction of cyclic adenosine-3', 5'-monophosphate (cAMP) production(125). This then acts through potential cAMP response elements in downstream areas (-1.4kb), which have been found to be PTH responsive in promoter-reporter assays (29;126). In both of these studies, the authors were unable to show any self-regulation of basal *CYP27B1* promoter activity and no VDREs were identified in the 1.4kb fragment. However, in each case $1,25(OH)_2D_3$ was unable to suppress PTH-induced transactivation. This suggests either that the *CYP27B1* gene promoter has an atypical VDRE, or that $1,25(OH)_2D_3$ achieves its effects by an indirect mechanism. These reports contrast with analysis of the murine promoter, which demonstrated both positive (PTH) and negative ($1,25(OH)_2D_3$) responsiveness in a region downstream of -0,9kb (127). In this study, calcitonin was shown to be a potent stimulator of *CYP27B1* expression, supporting previous reports in which calcitonin was shown to stimulate *CYP27B1* mRNA and enzyme activity under

normocalcemic conditions (128). This suggest that calcitonin (a noncollagenous protein secreted by osteoblasts), acting via distal areas of the nephron, may play an important role in the 'fine-tuning' of serum $1,25(OH)_2D_3$ levels during D_3 sufficiency.

Amongst the most prominent inhibitors of D_3 1α-hydroxylase are calcium, phosphate, and $1,25(OH)_2D_3$ itself. It seems likely that many of these effects are mediated indirectly through modulation of PTH production and secretion. However, as a consequence of the tight regulation of $1,25(OH)_2D_3$ production, analysis of the precise mechanisms involved in controlling the D_3 1α-hydroxylase has proved difficult, due to the very low enzyme levels.

1.2.4 VITAMIN D METABOLISM IN PERINATAL DEVELOPMENT

All three D_3 metabolites $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ circulate in mammalian fetuses. The cord concentrations of all three metabolites are consistently lower than those measured in the mother's serum. However, $24,25(OH)_2D_3$ is the dominant metabolite formed in fetoplacental tissues (129). Moreover, fetomaternal relationships of $1,25(OH)_2D_3$ concentrations are quite complex. In most studies, no correlation between fetal and maternal concentrations has been observed (130;131), whereas in the study by Ross *et al.*, (132), a highly significant correlation in both full term and preterm sheep was described.

Studies show that most of the $1,25(OH)_2D_3$ in fetal plasma is due to the fetal kidney production of the hormone (133;134), suggesting fetal control and autonomy in hormone regulation. Indeed, cord blood concentrations of $1,25(OH)_2D_3$ in fetal plasma from infants with Potter syndrome (renal agenesis) are one third those observed in healthy newborns. In addition, hepatic *CYP27A* mRNA levels have been detected in 17 to 19 week old human fetuses (135), further implying the possibility of fetal autonomy in D metabolism. However, a maternal contribution to fetal $1,25(OH)_2D_3$ cannot be eliminated, since radioactive $1,25(OH)_2D_3$ administration to pregnant monkeys (136), sheep (137) and rats (138) can be detected in fetal tissues. Parathyroid related peptide (PTHrP) is an important autocrine and /or paracrine growth factor regulator in fetal development. Its presence has been demonstrated in the placenta and fetal tissues in various species (139;140). In addition, it is implicated in the transfer of calcium across the placenta (139).

With respect to calcium homeostasis, the fetal-placental unit has a remarkable ability to meet its needs irrespective of maternal calcium or calciotropic hormones (141;142). Specifically, the fetal-placental unit has adapted to rapidly extract calcium from the maternal blood stream in sufficient amounts to mineralise the fetal skeleton in late gestation (143;144). Indeed, the fetus has a higher blood calcium than the ambient maternal calcium level (130;145). Furthermore, the newborns of hypocalcemic D-depleted female rats are normocalcemic (unpublished results from our laboratory), and newborn VDR-/- mice are normocalcemic until the time of weaning (146).

1.2.5 Gender differences in vitamin D metabolism

A microsomal D 25-hydroxylase, CYP2C11, is known to exist in male rat liver microsomes but not in those of females (147). This observation may well explain why microsomes (or P450 purifies from the microsomal fraction) obtained from female livers have consistently been shown to exhibit significantly far less activity in C-25 hydroxylation of D_3 than preparations obtained from male counterparts (148-150).

Furthermore, estrogen deficiency leads to a decrease in serum $1,25(OH)_2D$ regardless of age (151;152). Estrogen replacement in postmenopausal women can increase both total and free serum $1,25(OH)_2D$ (152;153), suggesting that menopause and the accompanying estrogen deficiency may remove an important trophic factor for the maintenance of serum $1,25(OH)_2D$ in aging women.

In parallel, serum total and free testosterone levels decrease with advancing age (154;155) and testosterone treatment has been shown to increase modestly both serum total and free $1,25(OH)_2D$ in hypogonadal men (156).

1.3 ROLE OF THE LIVER IN VITAMIN D_3 HOMEOSTASIS AND METABOLISM

1.3.1 HEPATIC STRUCTURE AND FUNCTION

The liver consists mainly of hepatocytes, the main parenchymal cell population. The non-parenchymal cells include sinusoidal, endothelial, Kupffer and stellate (Ito) cells. The hepatocytes represent 60% of the cell population. They are, however, more voluminous than other cells and consequently make up 80% of the parenchymal volume (157). The hepatocytes are organised in three-dimensional structures called acini (158). The hepatic acinus represents a structural and functional unit of the hepatic parenchyme whose main function consists of regulating the metabolism of the various substances exported to the systemic circulation (159).

The liver's microcirculation is composed of sinusoids, which are specialized capillaries whose discontinuous basal membrane is bordered by the sinusoidal cell population mentioned above. The sinusoidal endothelial cells form a selective semi-permeable barrier between the blood and the hepatic parenchyme, as described by De Zanger and Wisse (160). The Kupffer cells' main role is to endocytose many substances originating from the systemic circulation such as endotoxins (161). There also exists an intercellular space between the hepatocyte's plasma membrane and the endothelial cells called the space of Disse. This space encompasses the perisinusoidal stellate cells, which metabolize and store vitamin A (162), and synthesize the hepatocyte growth factor (HGF) (163). Stellate cells are also responsible for the synthesis of collagen, thus preventing cirrhosis of the liver.

The cells in zone 1 (or periportal area) are situated close to the supplying vessels and are bathed by blood of a composition similar to that in the afferent vessels. The cells in zone 3 (or perivenous area) are situated at the microcirculatory periphery of the acinar unit and receive blood that has already exchanged gases and metabolites with cells in zones 1 and 2 (midzonal).

1.3.2 VITAMIN D_3 UPTAKE: REGIONALISATION ALONG THE HEPATIC ACINUS

There exist two distinct mechanisms that can provide D_3 to the body. First, synthesis from UV light-dependent biosynthetic sites in the skin and secondly, through intestinal absorption from dietary sources of the vitamin.

Following these initial steps, efficient hepatic delivery of D_3 is necessary for the important C-25 hydroxylation step to occur (164). The production of 25(OH)D_3 is best served by a steady delivery of substrate, since rapid or pulse delivery leads to less 25(OH)D_3 production due to the likely overspill of D_3 to esterification enzymes, and possibly to extra-hepatic lipid storage sites (165;166).

Chylomicrons play an important role in D_3 transport, since they transport the vitamin after intestinal absorption (167). Following an i.v. injection of D, the vitamin is initially bound to lipoproteins (168) before transferring to the DBP (169), suggesting that D_3 is partly delivered to the hepatocytes via the DBP.

Although remnants or low density lipoproteins (LDL) have been shown to optimize uptake of D *in vitro* (170), manipulation of the diet in order to obtain different proportions of putative D_3 carrier proteins *in vivo* were found not to significantly affect the hepatic extraction of D_3 (171), an observation also made *in vitro* by Ziv *et al.* (172).

The hepatic handling of D_3 has been studied by many laboratories and most studies have reported that the fractional hepatic uptake of D varies between 40 to 60% in adult normal or D-depleted rats or dogs (171;173;174). The liver also has a considerable uptake capacity for the hormone 1,25$(OH)_2D_3$, which is estimated to be between 19.7 and 34% (175;176). Moreover, the hepatic extraction of D_3 has been shown to be independent of its hepatic venous or arterial route of delivery (175) and its hepatic clearance is estimated at 357ml/min in dogs (176). Studies in which total uptake has been investigated for periods varying from 18 sec to 70 min have revealed that the liver does not accumulate significantly more D_3 than that observed during the first pass across the organ (177). In addition, data indicate that there is no stringent regulation of uptake by the D_3 status (178).

Both regions of the acinus are equally able to extract D_3 which implies that the C-25 hydroxylation of the vitamin would, due to the concentration gradient across the hepatic acinus predominate in hepatocytes of the periportal area under normal basal conditions (179).

In addition, both the hepatocytes and the nonparenchymal cells of the rat liver have a high capacity to take up D_3 both *in vivo* and *in vitro* (174). The further metabolism to 25(OH)D_3 however, takes place almost exclusively in hepatocytes (174). It is postulated that the nonparenchymal cells may serve as a site of storage for the D_3; when the need for more 25(OH)D_3 arises, the stored D_3 might subsequently be transferred to the hepatocytes for

hydroxylation.

CYP27A is heterogeneously distributed within the liver acinus, with higher perivenous than periportal mRNA levels (58). Interruption of the enterohepatic circulation by a bile salt sequestrant increases the production of 27-hydroxycholestrol, steady-state *CYP27A* mRNA level and gene transcription (57;58). This data clearly indicates that *CYP27A* is subject to feedback inhibition at the transcriptional level by bile acids returning via the portal blood. Whether malabsorption-induced D_3 depletion leads to a similar regulation of the 25-hydroxylation of D_3 as that observed on the 27-hydroxylation of cholesterol following bile acid depletion (57) remains to be investigated. Moreover, the action of bile acids on the total hepatic output of $25(OH)D_3$ is unclear, since both the absence (180) and the presence of an effect (181) have been observed.

The anabolic function of the liver is particularly important since, under normal physiological conditions, it is the only organ involved in the C-25 hydroxylation of D_3 (164) giving rise to the most abundant circulating form of D_3 in normal individuals, $25(OH)D_3$. The circulating levels of $25(OH)D_3$ are the best indicator of the nutritional status of the vitamin. The liver is also involved in the biliary excretion of the main circulating D_3 metabolites such as $25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ (171;173) as well as many degradation products of D_3 metabolism (182). The liver can thus be considered as an organ involved in the whole body homeostasis of the D nutritional and endocrine systems through its anabolic as well as excretory functions.

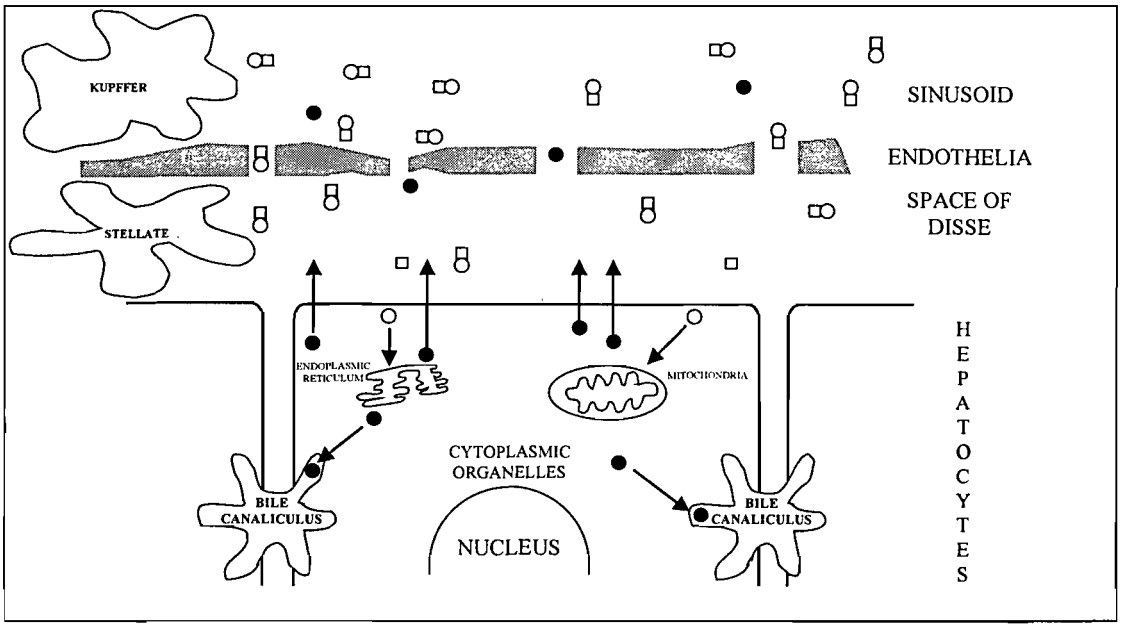

Figure 1.4 How the liver handles vitamin D and its metabolites. Vitamin D (D) and its metabolites (○) exist in blood largely bound to proteins (lipoproteins, chylomicrons, and D binding protein (□)). Bound metabolites can enter the space of Disse through fenestrations in the sinusoidal endothelium. Bound metabolites can then enter the hepatocyte and be converted to more water-soluble metabolites (●) by enzymes present in the endoplasmic reticulum or mitochondria. The metabolites are then either regurgitated back into the sinusoidal blood or are sorted to the biliary canaliculus. Cell sizes illustrated are not drawn to scale. Hepatocytes have a mean diameter of 17-18μM, whereas Kupffer, endothelial and bile duct cells have diameters of 11, 8 and 6μM respectively.

1.3.3 The liver: a non-classic vitamin D responsive organ

Evidence for a role for $1,25(OH)_2D_3$ and other parameters of calcium homeostasis in the liver began with studies of their requirements in response to hepatotoxic injury in liver regeneration (183). Extensions of these studies have shown that $1,25(OH)_2D_3$ is required for DNA synthesis after partial hepatectomy (184;185). $1,25(OH)_2D_3$ is also required for mitosis and compensatory liver growth (186) and is associated with an efficient transit across the G1 phase of the cell cycle (187). In addition, $1,25(OH)_2D_3$ induces several DNA replication enzymes, including DNA polymerase α (184;185), ribonucleotide reductase and probably thymidylate synthase (185). cAMP-dependent protein kinases are involved (184) in, but not solely responsible for, the $1,25(OH)_2D_3$ effects (184-186). Furthermore, the hormone has also been shown to stimulate the hepatic glycogen synthetic pathway and the synthesis of transferrin (188). Interestingly, the absence of actinomycin D inhibition of the latter effect has led to the suggestion that it occurs via a nongenomic pathway (189). To date, controversial data exists concerning the presence of VDR in the liver, with some investigators reporting its presence (190;191), whereas others doubt its importance (192).

1.3.3.1 Presence of vitamin D receptor in the liver

Thus, while some authors using biochemical and immunocytochemical techniques detected VDRs in liver (193;194), others failed to find detectable levels (195), and some used liver as a negative control of VDR gene expression (196;197). However VDR has been recently detected using a combination of sensitive molecular biological techniques, such as RT-PCR, with standard immunocytochemical methods in order to clarify VDR expression in rat liver(190;191).

1.4 THE INTESTINE: A CLASSIC VITAMIN D RESPONSIVE TISSUE

1.4.1 ANATOMY AND FUNCTION OF THE SMALL INTESTINE

The primary function of the small intestine is to absorb nutrients, electrolytes and water. This is achieved by mixing food with digestive enzymes to increase the contact of chyme with the absorptive cells of the mucosa. Absorption and movement of the contents are brought about by the activities of the absorptive cells of the mucosa and by the coordinated contraction of the smooth muscle cells of the muscularis extern (198;199). In addition to this fundamental role, a secondary function of the small intestine arises from the fact that it is also a major route of entry into the body for many xenobiotics including drugs.

The small intestine is divided arbitrarily into three parts: duodenum, jejunum and ileum. These regions are not anatomically distinct although there are differences in their absorptive and secretory capabilities. All three regions share a common histological pattern. Their wall, from inside outward is composed of the mucosa, the submucosa, the muscle layers and the serosa. The serosa is an extension of the peritoneum and consists of a single layer of flattened mesothelial cells overlying some loose connective tissues. The muscularis has an outer longitudinal layer and an inner circular layer of muscle. The submucosa is composed of a network of loose connective tissue rich in small blood vessels, lymphatics and nerve plexus. Furthermore, the mucosa has three components: a superficial lining of epithelium, the lamina propria, and the muscularis mucosa.

The epithelium is the innermost layer of mucosa facing the lumen of the bowel and consists of a single layer of columnar epithelial cells (enterocytes), which line both the crypts and the villi. Unlike hepatocytes, which regenerate only when death occurs, epithelial cells of the intestinal mucosa have a programmed limited lifespan. The villus epithelial cells are functionally mature and nondividing, whereas the crypt cells are immature and evolving. The crypt cells continue to mature as they ascend toward the villus and are extruded at its tip. The time required for migration from the base to the tip has been estimated to be 2 to 6 days (200). Interestingly, the rapidity of enterocyte migration and maturation may provide a protective mechanism against carcinogenic toxins (201).

1.4.2 ONTOGENY OF THE SMALL INTESTINE

In the human, the intestinal tract begins to develop from the premature entoderm and the surrounding splanchnic mesoderm at the end of the second week of gestation. A tubelike

structure is formed, and by day 18 the primitive gut is lined with non differentiated cuboidal cells which proliferate, obliterate the lumen, and then the intestine is recanalized (202). Homeotic genes (hox genes) regulate the patterning of the gastrointestinal tract. Indeed, regional differences in expression of hox genes in the mouse intestine have been demonstrated (203). The hox genes are critical early regulators of proximal to distal organ-specific patterning in mammalian gastrointestinal development.

During the fourth and fifth week of gestation the intestine elongates more rapidly than the embryo does and begins to form a loop which protrudes into the umbilical cord. At this stage the duodenum can be recognized by its contiguity with the cranial limb of the intestinal loop. Between the fifth and sixth week of gestation, the small intestine rotates around the axis of the superior mesenteric artery, moving counterclockwise. Further rapid elongation and coiling beyond the capacity of the slower growing abdominal cavity force the bulk of the developing intestine into the umbilical cord. At about 10 weeks of gestation the intestine re-enters the abdominal cavity and by the seventh to eighth week villi begin to form in the duodenum and proximal jejunum. Between 9 and 20 weeks of gestation, the human gut acquires many of the morphological and functional characteristics found in the adult (204). Furthermore, the highest epithelial proliferative activity is recorded between 9 and 20 weeks of gestation (205). The epithelium becomes columnar, and by the 12th week of gestation a single layer of epithelial cells lines the villi (206). After the 14th week the entire small intestinal mucosa is lined by villi. Primitive crypts begin to appear between 10 to 12 weeks and between the 5th and 40th week of gestation the intestine elongates approximately 1000-fold.

By the time of birth the intestine has acquired the digestive and absorptive functions necessary to cope with the neonatal diet, the maternal milk (207). The intestinal mucosa of the neonate displays a high level of structural development characterized by villi lined with a single layer of columnar epithelial cells, which have their absorptive well defined brush border (208).

In the rat, the length of the gut achieves 90% of its adult value by day 40, whereas body weight increases in a linear fashion (209). Between 20 and 40 days of age, the body weight of the animals continues to rise, but the ratio of the small intestine to body weight falls. It is well established, by morphological and biochemical criteria, that the human fetal intestine

is more mature at term than that of commonly examined mammalian models.

1.4.3 DRUG METABOLIZING ENZYMES IN THE SMALL INTESTINE

Numerous metabolic reactions occur in the gut wall, including those typically referred to as phase 1, and phase 2 processes. Almost all of the drug metabolizing enzymes present in the liver are also found in the small intestine, although their levels generally are much lower in the latter than in the former. Kinetically, the rate of intestinal metabolism of a drug is determined by the content of a particular catalytic enzyme within the enterocytes and the intracellular residence time of the drug subject to biotransformation.

Unlike the liver, in which the distribution of the P450 enzymes is relatively homogeneous (210), the distribution of these enzymes is not uniform along the villi within a cross-section of mucosa. Both the content and activity of P450s is higher in the proximal than in the distal small intestine (211). P450 content varies along the villus in rats, where P450 content at the villus tip is approximately 10 fold higher than at the crypts (211). Consistent with the P450 enzyme protein levels, the enzyme activities of P450 isoforms also are higher in the liver than in the small intestine.

1.4.4 BIOLOGICAL ACTIONS OF VITAMIN D_3

The small intestine is a major target of $1,25(OH)_2D_3$. In general, these actions take several hours and are mediated by the VDR. However, some effects of $1,25(OH)_2D_3$ are rapid, may involve a membrane-bound rather than a cytosolic VDR, and may be mediated by the PKC pathway (212).

The most critical role of $1,25(OH)_2D_3$ in mineral homeostasis is to enhance the efficiency of the small intestine to absorb dietary calcium and phosphate as demonstrated conclusively by studies in the VDR null mice (213). In the absence of VDR, normalization of circulating levels of calcium and phosphorus through dietary supplementation corrects most of the phenotypic features of D_3 resistance including parathyroid gland growth, bone mineralization, and growth plate histology. These findings concur with prior clinical observations in patients with vitamin D-resistant rickets whose bone abnormalities were

resolved by calcium infusions.

$1,25(OH)_2D_3$ increases the entry of calcium through the plasma membrane in the enterocyte, followed by the movement of calcium through the cytoplasm, and the transfer of calcium across the basolateral membrane into the circulation. $1,25(OH)_2D_3$ is the only hormone known to stimulate intestinal calcium transport directly. Other D_3 metabolites can stimulate calcium transport, but only at higher doses, consistent with their lower affinity for the VDR. The mechanism for stimulation of transcellular calcium transport is not entirely clear, but induction of a cytosolic calcium-binding protein (calbindin D) and the basolateral calcium pump undoubtedly are important components (214). Increasingly, evidence suggests that the VDR-mediated effects of $1,25(OH)_2D_3$ may not be the only mode of action by which the hormone stimulates calcium absorption by the enterocyte. Rapid effects of $1,25(OH)_2D_3$ appear to mediate an increase in both the vesicular and paracellular pathways for intestinal calcium absorption. The actual contribution of these nongenomic pathways to intestinal calcium absorption *in vivo* is unclear.

In addition to its effects on calcium absorption, $1,25(OH)_2D_3$ increases active phosphate transport. However, significant phosphate absorption also occurs in $1,25(OH)_2D_3$-deficient states (215). The sterol directly stimulates the expression of the Na-Pi cotransporter (216) and affects the composition of the enterocyte plasma membrane, increasing fluidity and phosphate uptake. Sodium-independent entry of phosphate occurs independently of D status (217). Little is known, however, concerning the molecular mechanisms involved in the extrusion of phosphate across the basolateral membrane into the circulation.

$1,25(OH)_2D_3$ has also been shown to alter the proliferation and differentiation of cell lines, including colon cells (218). Most of these effects are mediated by the cytosolic VDR. However, some effects of $1,25(OH)_2D_3$ are rapid, may involve a membrane-bound rather than a cytosolic VDR, and may be mediated by the PKC pathway (219).

An important action of $1,25(OH)_2D_3$ in the intestine is to increase intestinal $1,25(OH)_2D_3$ 24-hydroxylase activity. The D_3-24 hydroxylase enzyme is found in tissues that are targets for $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ production, as discussed previously. It is thought to be the first step in the degradation of $1,25(OH)_2D_3$. Thus, the activity of the D_3-24 hydroxylase may regulate the action of $1,25(OH)_2D_3$ in the intestine. $1,25(OH)_2D_3$ markedly increases the mRNA levels of *CYP24* in the intestine of intact animals (88;96) as well as in intestinal

cell lines (220;221).

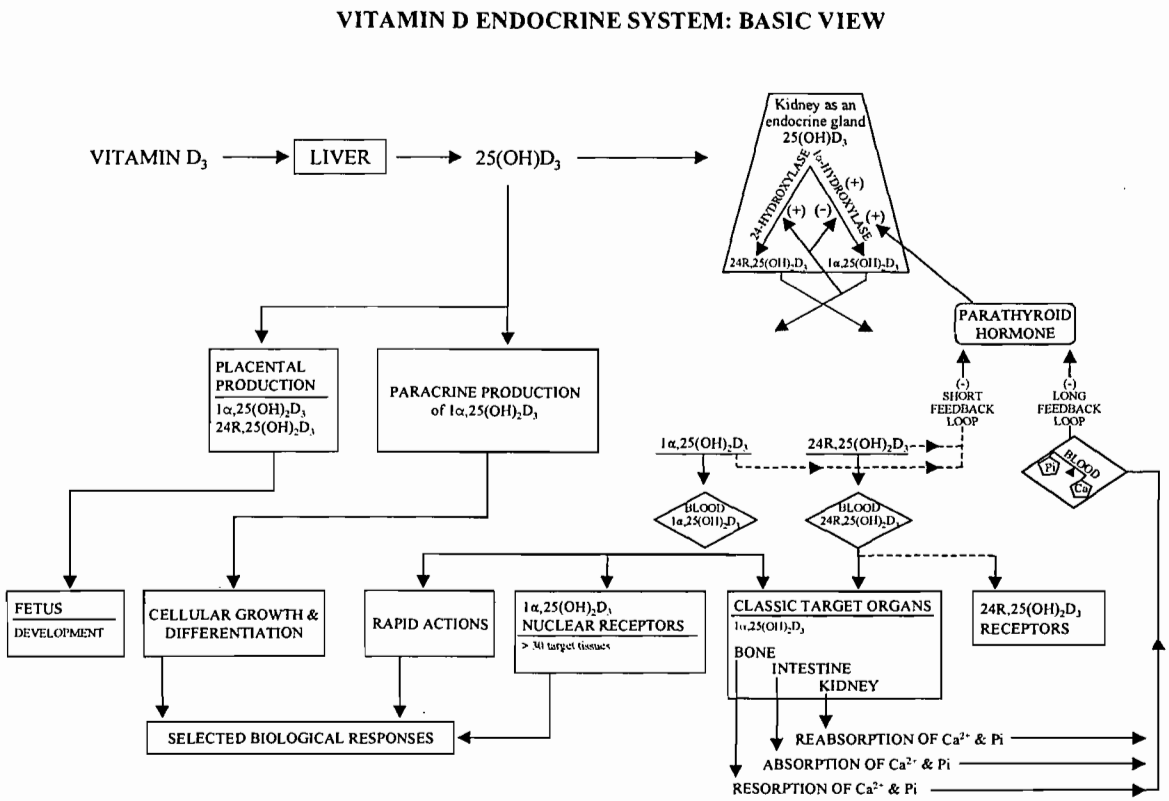

Figure 1.5 Overview of the vitamin D system.

CHAPTER 2: EXPERIMENTAL SECTION (STUDIES IN LIVER)

2.1 EFFECT OF THE VITAMINE D_3 HORMONAL AND NUTRITIONAL STATUS ON CYP27A, THE HEPATIC VITAMIN D_3 25-HYDROXYLASE

2.1.1 Preliminary findings

It has long been established that the liver is the primary and main organ involved in the hydroxylation of D_3. The C-25 hydroxylation of D_3 has been shown to occur in both the mitochondria and endoplasmic reticulum. Previous studies have shown $1,25(OH)_2D_3$ inhibits the *in vitro* synthesis of $25(OH)D_3$ by liver homogenates and perfused liver from rachitic rats (222). In addition, *in vivo* experiments indicate that in both humans and animals, $1,25(OH)_2D_3$ administration is associated with decreases in serum $25(OH)D_3$ concentrations (223-225). To date, regulation studies on the *CYP27A* gene, the mitochondrial D_3 25-hydroxylase, have focused primarily on bile acid metabolism. Studies in rat (both *in vivo* and *in vitro*) have shown that *CYP27A* is downregulated at the transcriptional level by hydrophobic bile acids (57;226;227). Furthermore, *CYP27A* in rat has been shown to undergo diurnal variation, upregulation by glucocorticoids and downregulation by insulin (43;57;59). No studies have examined the effect of the D_3 hormonal and/or nutritional status on the hepatic *CYP27A*.

2.1.2 Hypotheses

Previous studies have indicated that $1,25(OH)_2D_3$ significantly reduces circulating $25(OH)D_3$ levels by inhibiting hepatic production of $25(OH)D_3$ (228). However, the molecular mechanisms involved in the regulation of hepatic production of 25(OH)D have not been addressed thus far. Since *CYP27A*, the mitochondrial D_3-25 hydroxylase, has been shown to be inducible by D_3 in keratinocytes *in vitro* (65), we hypothesize that regulatory mechanisms related to the D_3 status may affect the *CYP27A* gene expression level in the liver.

2.1.3 Objectives

- Evaluate the effect of the D_3 hormonal and/or nutritional status on the rat hepatic *CYP27A* gene transcript.
- Evaluate the steady state expression level of the *CYP27A* in human liver and kidney specimens.

2.2 RESULTS

2.2.1 Article 1: High sensitivity of the rat hepatic vitamin D_3 25-hydroxylase CYP27A to 1,25-dihydroxyvitamin D_3 administration.

Article in press, American Journal of Physiology.

(ARTICLE 1)

HIGH SENSITIVITY OF THE RAT HEPATIC VITAMIN D_3-25 HYDROXYLASE *CYP27A* TO 1,25-DIHYDROXYVITAMIN D_3 ADMINISTRATION

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ABSTRACT

CYP27A is considered the main vitamin D_3 (D_3)-25 hydroxylase in humans. Our purpose was to evaluate the effect of the D_3 nutritional and hormonal status on hepatic *CYP27A* mRNA, cellular distribution, transcription rate and enzyme activity. Studies were carried out in normal and in D-depleted rats supplemented with D_3, $25OHD_3$ or $1,25(OH)_2D_3$. *CYP27A* exhibited a significant gender difference and was observed throughout the hepatic acinus not only in hepatocytes but also in sinusoidal endothelial, stellate and Kupffer cells. Neither D_3 nor $25OHD_3$ influenced *CYP27A* mRNA levels. However, $1,25(OH)_2D_3$ repletion led to a 60% decrease in *CYP27A* mRNA which was accompanied by a 46% decrease in mitochondrial D_3-25 hydroxylase activity. The effect of $1,25(OH)_2D_3$ was mediated by a significant decrease in *CYP27A* transcription while its mRNA half-life remained unchanged. Our data indicate that *CYP27A* is present in hepatic parenchymal and sinusoidal cells, and that the gene transcript is not influenced by the D_3 nutritional status but is transcriptionally regulated by $1,25(OH)_2D_3$ exposure.

Key words: CYP27A, vitamin D_3, $25OHD_3$, $1,25(OH)_2D_3$, bile acid biosynthesis, Kupffer cells, stellate cells, hepatocytes, sinusoidal endothelial cells, D_3 25-hydroxylase.

INTRODUCTION

The secosteroid vitamin D_3 (D_3) of endogenous or exogenous origin has, in its native form, no biological activity. Once in circulation, D_3 is efficiently taken up by the liver (26) and hydroxylated at C-25 by a mitochondrial mixed function oxidase CYP27A (C_{27} sterol hydroxylase (EC 1.14.13.15)) (15). In humans, the enzyme is presumed to be the only D_3-25 hydroxylase (51). However, a microsomal D_3-25 hydroxylase has also been reported in rodents (11), chickens (12) and pigs (35) but only the porcine enzyme (which has been termed *CYP2D25*) has been cloned to date (34;44).

CYP27A is a cytochrome P450 that catalyses the first step in the oxidation of the cholesterol side chain in the secondary "acidic" bile acid biosynthesis pathway (13). CYP27A is also able to hydroxylate D_3 and D_3 metabolites at position C-25 (51) as well as at other positions on the secosteroid side chain (29;54). It has also been reported to be able to catalyse the 1α-hydroxylation of 25-hydroxyvitamin D_3 ($25OHD_3$) albeit at a much lower rate than the transformation of D_3 into $25OHD_3$ (3). However, unlike the tight regulation by the D_3 endocrine system associated with the renal 25-hydroxyvitamin D_3-1α-hydroxylase, the sensitivity of the gene encoding *CYP27A* to D_3 or to D_3 metabolites has not been characterized. The presence of regulatory mechanisms related to the D_3 status as a modulator of the 25-hydroxylation of the vitamin is, however, a widely accepted notion which rests on the studies of DeLuca's group in the early 1970s (10;12). Several laboratories have attempted to evaluate the mechanism(s) involved in the regulation of the activity of the D_3-25 hydroxylase. In the early 1980s, two independent reports (5;8) raised the possibility that $1,25(OH)_2D_3$ might be an inhibitor of the enzyme

in rats as well as in humans. Indeed, Bell *et al.* (8) reported that D_3 administration to human subjects significantly increased mean serum $25OHD_3$ whereas the concomitant administration of $1,25(OH)_2D_3$ completely prevented the increase in serum $25OHD_3$ in response to the same dose of D_3. Subsequent studies revealed that the response of the D_3-25-hydroxylase to various challenges *in vitro* was greatly influenced by the *in vivo* calcium and/or D_3 status of the animals (9). However, supplementation with $1,25(OH)_2D_3$ was shown to influence the *in vivo* handling of D_3 by accelerating its biotransformation as well as by increasing the metabolic and biliary clearances of D_3 and/or D_3 metabolites (18;24;31;32). The latter studies suggest the presence of a $1,25(OH)_2D_3$-mediated increase in the utilisation of the substrate and/or in its turnover but do not, in any way, directly address the effect of $1,25(OH)_2D_3$ on the hepatic mitochondrial D_3-25 hydroxylase *CYP27A*.

To date, studies examining the regulation of the gene encoding *CYP27A* have focussed on its role as a mixed function oxidase involved in bile acid biosynthesis (56). The aim of the studies was, therefore, to investigate the influence of the D_3 nutritional and endocrine status on *CYP27A* as a D_3-25 hydroxylase in the rat liver. We now report that $1,25(OH)_2D_3$ is a major regulator of the hepatic *CYP27A* which translates into significant decreases in *CYP27A* steady state mRNA levels and transcription rate, as well as into a significant decrease in the mitochondrial C-25 hydroxylation of 1α-hydroxyvitamin D_3.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The influence of the D_3 endocrine system on the handling of the gene encoding the hepatic mitochondrial D_3-25 hydroxylase *CYP27A* was evaluated in D depleted rats, and in D depleted rats following *in vivo* repletion with physiological concentrations of either D_3, $25OHD_3$ or $1,25(OH)_2D_3$. D depletion as well as all repletion procedures were carried out as previously reported (59). The specific end points of the studies included evaluation of the *i)* gender differences, and response to cytochrome P450 inducers on *CYP27A* mRNA levels, *ii)* presence of *CYP27A* in the main hepatic cell populations, and *iii)* effect of $1,25(OH)_2D_3$ on *CYP27A* steady state mRNA levels, mRNA half-life, transcription rate, and the mitochondrial CYP27A hydroxylation activity at C-25.

A *CYP27A* gene fragment corresponding to base pairs 399 to 803 of the N-terminal coding region of the *rCYP27A* sequence of Su *et al.* (58) (GenBank, accession number M38566) was generated by RT-PCR as previously reported (59). Northern blot analyses of rat livers hybridized with the *rCYP27A* gene fragment generated consistently revealed a single band of 2.3 kb.

Animals were treated according to the standards of ethics for animal experimentation of the Canadian Council on Animal Care and all protocols were approved by the local animal ethics committee.

REPLETION WITH D_3, $25OHD_3$, OR $1,25(OH)_2D_3$

Expression of the hepatic *CYP27A* gene transcript was studied in normal control rats fed a commercial rat chow diet (Harlan Tekland Global Diet, Madison, WI, USA), D depleted animals fed a semi-synthetic diet as described previously (30), and in animals repleted with D_3, $25OHD_3$, or $1,25(OH)_2D_3$ but kept on the D depleted diet through out the repletion procedure. The repletion protocols were identical to those used to evaluate the effect of the D_3 status on the intestinal *CYP27A* (59). Briefly, all compounds were administered by intraperitoneal (i.p.) osmotic mini-pumps (Alza Corporation, Palo Alto, CA, USA) containing either D_3 (6.5 nmol/day (low dose), or 32.5 nmol/day (high dose)), $25OHD_3$ (28 pmol/day), or $1,25(OH)_2D_3$ (28 pmol/day). At the time of osmotic mini-pump implantation a loading dose of 3.2 (low dose), 16.2 (high dose) nmol D_3, 14 pmol $25OHD_3$, or 14 pmol $1,25(OH)_2D_3$ was administered to rapidly raise serum concentrations of D_3, $25OHD_3$, or $1,25(OH)_2D_3$ and hence accelerate the establishment of steady state conditions. Animals were killed 1, 3, 5 or 7 days following initiation of the repletion protocols. The serum ionized calcium, and vitamin D_3 metabolites concentrations achieved under all repletion protocols (except for the high dose of D_3) have been previously reported (59).

TREATMENT WITH CYTOCHROME P-450 INDUCERS

Studies on the induction of the gene encoding *CYP27A* were achieved using xenobiotics known to induce cytochrome P-450 izozymes. Normal male rats were exposed to either *i)* dexamethasone (two daily i.p. injections, 100mg/kg), *ii)* 3-methylcholanthrene (one single i.p. injection, 30 mg/kg), *iii)* β-naphtoflavone (three daily i.p. injections, 80mg/kg), *iv)* acetone (1% v:v in drinking water) for a period of 10 days,

or *v)* phenobarbital (350 mg/ml in drinking water) for a period of 10 days (45).

EXPERIMENTAL PROCEDURES

DETERMINATION OF CIRCULATING Ca^{2+} AND D_3 METABOLITES

Serum Ca^{2+} concentrations were measured with an ICA2 ionized Ca^{2+} analyzer (Radiometer, Copenhagen, Denmark). Serum $25OHD_3$ and $1,25(OH)_2D_3$ concentrations were measured using the IDS $25OHD_3$ and $1,25(OH)_2D_3$ assay Kits (IDS, Boldon, Tyne and Wear, UK) according to the manufacturer's instructions.

HEPATIC CELL ISOLATION

At the time of euthanasia, the livers were flushed with saline and processed for isolation of the individual cell populations. Cells used for RNA analysis were placed in Trizol solution (Burlington, ON, Canada) and RNA was extracted as described by the manufacturer.

Hepatocytes were isolated from non-fasting animals as mentioned elsewhere (25). Rat sinusoidal cells were isolated by the method of Knook and Sleyster (37) with the following modifications. After Metrizamide density gradient, cells were washed in GBSS, pH 7.4 at 4°C, resuspended, and introduced in a type J2-21M centrifuge (Beckman Instruments, Palo Alto, CA) equipped with a JE-6B elutriation rotor and a Sanderson chamber. While centrifugation at 2500 RPM, cells were washed out at pump flows of 13, 23, and 42 ml/min to collect stellate, endothelial and Kupffer cells respectively using GBSS, pH 7.4 at 4°C. Cells were centrifuged, counted and viability evaluated. Sinusoidal cells had a viability greater than 95% and were freed of hepatocytes. Cell populations were identified by immunocytochemistry and found to be >88% pure (43).

Cell viability and yield were evaluated by the Trypan blue exclusion test and by counting viable cells in each cell population respectively.

NORTHERN BLOT ANALYSIS

At the time of euthanasia, the livers were isolated and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total liver RNA was extracted, blotted onto nylon membranes (Qiagen, Mississauga, ON, Canada) and processed for Northern analyses using the radiolabeled 404 base *rCYP27A* cDNA probe generated in our laboratory (59).

RT-PCR

After Dnase treatment, 1 μg of total RNA from sinusoidal cells was converted in cDNA (First-Strand cDNA synthesis Kit) using $pd(N)_6$ as primer; 2.0μL of RT reaction was amplified for either 25 (*CYP27A*) or 20 cycles (*GAPDH*) using specifics primers and Taq PCR Master Mix and 0.1 μL of [α-^{32}P] dCTP (3 000 Ci/mol) in a Touchdown Thermal Cycling system (Hybaid, Teddington, U.K.). Design of primers to generate *rCYP27A* and *GAPDH* cDNA fragments was made with the Primers Software of Williamstone Enterprises (http://www.williamstone.com) and the sequence of Su *et al.* (58) (*CYP27A*) and Tso *et al.*(62) (*GAPDH*). cDNA PCR products were loaded and separated onto a non-denaturing 8% polyacrylamide TBE gel. The gel was dried and exposed to Kodak X-Omat AR film at -80°C in the presence of an intensifying screen for 4 to 16 hours. Densitometry was performed as described previously. (20;38).

HALF-LIFE OF THE *CYP27A* GENE TRANSCRIPT

Studies on the half-life of the *CYP27A* gene transcript were achieved in D depleted rats subjected to i.p. injections of 0.5mg/kg actinomycin D dissolved in 95%

ethanol:saline (1:1 v:v) administered 6, 12, 18 and 24 hours before sacrifice. In studies carried out in $1,25(OH)_2D_3$ injected (12 nmol/kg, i.v.) animals, actinomycin D was first administered half an hour before $1,25(OH)_2D_3$ and subsequently every six hours over a 24 hour-period. $1,25(OH)_2D_3$ continued to be administered every 6 hours. *CYP27A* mRNA levels were evaluated as described above.

NUCLEAR RUN-ON TRANSCRIPTIONAL ASSAY

Nuclei were isolated from hepatic of D-Ca- or of $1,25(OH)_2D_3$ repleted rats by the method of Widnell and Tata (67) using successive sucrose gradient centrifugations. The rate of *CYP27A* gene transcription was measured using a previously described nuclear run-on transcriptional assay (50) with the modifications described in Theodoropoulos *et al.*(59). The labelled RNA was hybridized to nylon membranes on which 300 ng of the 404 bp D_3-25 hydroxylase cDNA fragment 150 ng of 18S ribosomal RNA cDNA fragment as a positive control, and 100 ng of pBS as negative control in hybridization solution (5% SDS, 400 mM $NaPO_4$ pH 7.2, 1mM EDTA, 1mg/ml BSA, 50% formamide and 240 μg/ml of salmon sperm DNA). The membranes were prehybridized for 4 hours at 52°C in hybridization solution without labelled RNA, then hybridization was performed at 52°C for 72 hours. The membranes were washed and exposed to x-ray films for 7 days and densitometry was performed as previously described (20;38).

IN SITU RT-PCR HYBRIDIZATION

Paraffin liver sections were mounted onto slides pre-treated with 3-aminopropyltriethoxysilane (APES), dewaxed in xylene and then rehydrated through a series of ethanol baths, and finally immersed in DEPC-treated water. Slides were

incubated as described in Gascon Barré *et al.*, 2001 (23).

In situ RT-PCR were performed by the method of Mee et al. (40) with the modifications previously described (23). The reaction was carried out in 25 µL with OneStep RT-PCR kit and 0.6µM *rCYP27A* specific primers (58) using a Hybaid thermal cycler provided with a *in situ* block (Hybaid, Teddington, UK). Sections were heated for 30 min. at 50°C, 15 min. at 95°C and then 10 cycles of 95°C for 30 sec., 62°C for 30 sec., and 72°C for 30 sec. were performed; finally slides were heated for 10 min. at 72°C. Sample were washed twice in PBS and fixed in 4% paraformaldehyde-PBS for 20 min. at 4°C and incubated with 0.25% acetic anhydride in 0.1 TEA for 10 min. and rinsed in 90% ethanol and allowed to dry.

An *in situ* hybridization was performed using the *rCYP27A* antisense riboprobes (using linearised *CYP27A* cDNA as template) were generated by the single strand RNA synthesis technique using T7 RNA polymerases and [α-^{33}P]UTP (800 Ci/mmol). Hybridization was performed at 42°C for 16 hours with 100μL of hybridization solution (50% formamide, 2XSSC, 1X Denhart's, 0.25M Tris-HCl, pH 7.5, 10% dextran sulfate, 0.5M Na pyrophosphate, 0.5% SDS, 25μg/mL denatured salmon sperm DNA, 250 μg/mL yeast tRNA) and 1 X10^7 cpm/mL of antisense. After washing, autoradiography was performed with NBT-2 emulsion (Interscience, Mississauga, ON, Canada). Slides were exposed for 5 days at 4°C, developed with D19 (Interscience, Mississauga, ON, Canada) developer and counterstained with hematoxylin and eosin.

MITOCHONDRIAL D_3-25 HYDROXYLASE ENZYME ACTIVITY

Liver from hypocalcemic D depleted and $1,25(OH)_2D_3$ treated rats (28pmol/d, for 7 days) were homogenized in 10 volumes of 0.25M sucrose, 1mM EDTA, 10mM Tris, 10M KCl, heparin 3U/ml, pH 7.4. Mitochondria were isolated according to Rosenberg and Kappas (48) with the modifications described previously (59). The final mitochondrial pellet was resuspended in 0.25M sucrose, 1mM EDTA, 10mM Tris, 10M KCl, heparin 3U/ml, pH 7.4.

Incubation reactions contained 0.5mg mitochondrial proteins suspended in 40mM potassium phosphate, 0.25M sucrose, 200mM EDTA, 20mM $MgCl_2$, pH 7.4, 0.2mg bovine serum albumin, 2μg N,N'diphenylphenylelenediamine (Aldrich Chem. Co., Milwaukee, WI) and 10mM isocitric acid (Sigma Chemicals, St.Louis, MO, USA). The enzyme reaction was started with 20 nmol 1α-hydroxyvitamin D_3 (Leo Pharma, Ajax, ON, Canada) and continued for 40 min at 37°C with gentle shaking. Control conditions were carried out using boiled mitochondria. The reaction was terminated with 3.75ml chloroform/methanol (1:2 v/v) and 6000 cpm $[^3H]1\alpha,25(OH)_2D_3$ was added to monitor recovery during the extraction and chromatographic procedures (16). After extraction and evaporation, the residue was dissolved in 150 μl hexane and injected into a Beckman model 160 fitted with an absorbance detector at 254 nm (Beckman Instruments, Palo Alto, CA). A Zorbax-Sil column (4.6 x 250mm) (Dupont Instruments, Wilmington, DE) was used. Elution was done in hexane /isopropanol (9:1 v/v) at a flow rate of 2ml/min. The fractions corresponding to authentic $1\alpha,25(OH)_2D_3$ (retention time 15 min, without overlap from $1\alpha OHD_3$), were collected and counted in a beta spectrometer (Beta LS1801, Beckman Instrument, Palo Alto, CA). Identity of the product was further confirmed by a

second HPLC on a C-18 column eluted with hexane:isopropanol (8:2, v:v).

STATISTICAL ANALYSES

Data are presented as means ± S.E.M. Statistically significant differences between group means were evaluated by ANOVA, or the Student's "t" test as indicated in the figure legends. Individual between-group contrasts were evaluated using the Bonferroni test.

RESULTS

PARAMETERS OF THE D_3 NUTRITIONAL AND ENDOCRINE STATUS

Table 1 presents the circulating concentrations of $25OHD_3$ and $1,25(OH)_2D_3$ as well as those of the circulating Ca^{2+} concentrations. Serum $25OHD_3$ concentrations were significantly increased in both D_3-supplemented rats compared to the D depleted, $25OHD_3$ and $1,25(OH)_2D_3$-repleted rats but both doses of D_3 lead to serum $25OHD_3$ within the normal physiological range 28 and 75 nmol/L in animals receiving the low and high doses respectively. Serum $1,25(OH)_2D_3$ concentrations increased in all repleted groups compared to the D-depleted controls. However, serum $1,25(OH)_2D_3$ concentrations were found to be significantly higher in both D_3 repleted groups than in those receiving either $25OHD_3$ or $1,25(OH)_2D_3$.

CELLULAR LOCALIZATION

Investigation of the intrahepatic cellular localization of the gene encoding *CYP27A* revealed that the transcript was expressed not only in hepatocytes but also in sinusoidal cells. Indeed, the *CYP27A* gene transcript was clearly found in freshly isolated sinusoidal endothelial, stellate (Ito) as well as in Kupffer cells as illustrated in Figure 1. However, hepatocytes exhibited the highest abundance of the *CYP27A* gene transcript with an averaged *CYP27A* mRNA levels of 53%, 23%, and 9% in sinusoidal endothelial, stellate, and Kupffer cells respectively compared to the levels found in hepatocytes (100%).

GENDER DIFFERENCES AND DRUG INDUCIBILITY

Evaluation on the gender differences in *CYP27A* mRNA levels reveals that

female rat livers exhibit a 43% higher levels of the gene transcript than their male counterparts ($p<0.01$) as illustrated in Figure 2A and B. In addition, the hepatic steady state levels of the *CYP27A* transcript were found to be significantly induced by the two classical cytochrome P-450 inducers dexamethasone (+45% over basal values) and β-naphtoflavone (+41% over basal values) (Fig. 2B and D). 3-methylcholanthrene, acetone, and phenobarbital did not significantly affect the steady state abundance of the *CYP27A* transcript.

EFFECT OF D_3, $25OHD_3$ OR $1,25(OH)_2D_3$

CYP27A mRNA levels during long-term exposure to D_3 or $25OHD_3$

Repletion of D depleted rats with either D_3 (low dose) or $25OHD_3$ was found not to significantly influence the liver *CYP27A* mRNA levels following 1, 3, 5 or 7 days of repletion as illustrated in Figure 3 for data obtained on day 7 of the repletion protocol. Rats fed the high dose of D_3 exhibited a transient 55% decrease in *CYP27A* mRNA levels at the 3 day time-point but not at any other time-points as illustrated for values obtained following one week of repletion. Serum calcium were normalized in all repleted groups and hypercalcemia was not observed in any of the groups.

CYP27A mRNA levels during long-term exposure to $1,25(OH)_2D_3$

By contrast, as illustrated in Figure 4, the hepatic *CYP27A* gene transcript was very sensitive to the continuous administration of $1,25(OH)_2D_3$ leading to normal circulating Ca^{2+} and $1,25(OH)_2D_3$ concentrations as previously reported (59). Indeed, *CYP27A* mRNA levels progressively decreased throughout the week of $1,25(OH)_2D_3$ repletion with a decrease of 23% compared to values observed in D depleted animals

after one day of repletion to attain a 60% decrease after 7 days of continuous $1,25(OH)_2D_3$ exposure ($p<0.0007$).

Intra-acinar localisation of the CYP27A gene transcript

Figure 5 presents data on the *in situ* hybridization of liver specimens obtained from D depleted, normal D replete controls and $1,25(OH)_2D_3$ repleted rats. As illustrated, in liver specimens obtained from D depleted (Fig. 5A and B) and from normal controls (Fig. 5D and E), the *CYP27A* gene transcript was found to be present throughout the hepatic acinus with *CYP27A* mRNA hybridization being observed in both the periportal and the perivenous regions of the acinus. The intensity of the *CYP27A* mRNA signal was found to be only slightly more intense in liver specimens obtained from D depleted than in those obtained from normal controls. After one week of $1,25(OH)_2D_3$ repletion, however, a clear decrease in the intensity of the *CYP27A* mRNA hybridization signal was observed in both periportal and the perivenous region of the hepatic acinus (Fig. 5G and H). Negative *in situ* hybridization controls using the *rCYP27A* sense riboprobes on hepatic specimens obtained from in D depleted, normal controls, and $1,25(OH)_2D_3$ repleted rats are presented in Figure 5C, F and I respectively.

Activity of the mitochondrial D_3-25 hydroxylase

Figure 6 illustrates the effect of one-week exposure to $1,25(OH)_2D_3$ on the hepatic mitochondrial D_3-25-hydroxylase activity. $1,25(OH)_2D_3$ repletion had a significant influence on CYP27A activity with an averaged 46% decrease in $1\alpha,25(OH)_2D_3$ production following incubation with $1\alpha OHD_3$ in liver mitochondria obtained form $1,25(OH)_2D_3$ repleted compared to those obtained from D depleted rats ($p<0.03$).

MECHANISMS OF $1,25(OH)_2D_3$ ACTION

CYP27A mRNA half-life

As illustrated in Figure 7, actinomycin D treatment of D depleted rats with 0.5mg/kg progressively decreased *CYP27A* mRNA levels throughout the 24h time-frame studied to nearly undetectable levels at the 24h time-point ($p<0.0001$). Under our experimental conditions, the half-life of the *CYP27A* gene product was estimated to be 12.7h. $1,25(OH)_2D_3$ administration was found to influence *CYP27A* mRNA levels in a manner similar to that observed in animals treated with actinomycin D alone. In addition, over the time-period studied, the concomitant administration of the hormone and actinomycin D did not significantly affect *CYP27A* mRNA levels over that observed with the hormone or actinomycin alone.

Transcription rate of the CYP27A gene

Nuclear transcription run-on assays were performed on nuclei isolated from livers of D depleted rats as well as on nuclei obtained from livers of D depleted animals exposed to a single 12 nmol/kg i.v. dose $1,25(OH)_2D_3$ 6h before euthanasia (Fig. 8). The *18S* ribosomal gene was used as control gene for both the untreated and treated groups. Quantification for the nuclear run-on assays indicated that within 6 hours of $1,25(OH)_2D_3$ exposure, the transcription rate of the gene encoding the *CYP27A* was decreased to nearly undetectable levels compared to the level of expression observed in control livers. Non-specific hybridization, estimated by hybridization to pBS plasmid DNA, did not account for the observed *CYP27A* mRNA decrease in transcription rate.

DISCUSSION

Our data show for the first time the presence of the *CYP27A* gene transcript in hepatic sinusoidal cells. Although, hepatocytes were found to harbor the highest level of the transcript, the observation indicates that cell populations other than hepatocytes may also be involved in the production of $25OHD_3$ in the normal rodent liver. These data indicate that *CYP27A* is more widely distributed than originally thought as the intestine, kidney, calvaria, long bones, lung, spleen, adrenals, epidermis, and the central nervous system have also been shown to express the *CYP27A* gene transcript (14;36;55;59;60). Circulating macrophages and vascular endothelial cells are also known to harbor the *CYP27A* gene product and to hydroxylate cholesterol at C-25 as a mean of excreting cholesterol (14). These observations indicate that although the liver is the main $25OHD_3$ production site under normal physiological circumstances, many organs and cell types also harbor the enzyme and hence possess the capacity to metabolize D_3 to $25OHD_3$ as clearly illustrated in previous studies on the rat duodenum and human fetal jejunum and colon (59;60).

The present studies also reveal a significant gender difference in the expression of the gene with a 43% higher steady state mRNA levels in female than in male rat livers. The latter observation is in agreement with the data reported by Andersson and Jornvall (2) and Saarem and Pedersen (52) where the activity of CYP27A was found to be higher in female than in male rats as well as those of Addya *et al.* (1) who reported that the enzyme was highly influenced by sex hormones. This observation is also in line with the higher expression of the gene in normal human liver specimens obtained from women

compared to those obtained from men (23). Interestingly, data on the intra-acinar distribution of the *CYP27A* gene transcript show that in D depleted as well as in normal rats, *CYP27A* mRNA was widely distributed within the liver parenchyma with a rather diffuse distribution all along the hepatic acinus. $1,25(OH)_2D_3$ repletion significantly decreased the *CYP27A* gene transcript as evidenced by both Northern blot analysis and *in situ* hybridization. The decrease in *CYP27A* mRNA was observed in both the periportal as well as the perivenous regions of the hepatic acinus and translated into a significant reduction in mitochondrial C-25 hydroxylation activity.

Hepatic *CYP27A* has previously been shown to be transcriptionally regulated by glucocorticoids, growth hormone, cholic acid, cyclosporine A, insulin and the physiological state of the animal (42;56;58;64-66). In the present studies, the gene, as expected (57), was found to be induced by dexamethasone, a known inducer of the CYP3A family of cytochrome P450s (28). In addition, *CYP27A* was shown to be upregulated by β-naphtoflavone, a known inducer of CYP1A1 and 1A2 (17). On the other hand, other classical cytochrome P-450 inducers (phenobarbital, acetone, ethanol) did not influence the abundance of the *CYP27A* gene transcript. Our data, however, clearly show that $1,25(OH)_2D_3$ led to a significant decrease in the transcription rate of the *CYP27A* gene without significantly affecting the stability of its message as evidenced by a similar decrease in *CYP27A* abundance in D depleted animals and in animals exposed to $1,25(OH)_2D_3$. The almost complete inhibition of *CYP27A* transcription within 6h after a single $1,25(OH)_2D_3$ i.v. dose suggests that the action of the hormone involves receptor interactions. The normal rat liver has been shown to harbor a low abundance of the nuclear vitamin D_3 receptor (VDR_n) (53) and the presence of a membrane receptor

(VDR_m) (7) has previously been suggested in rat hepatocytes (4;6). However, the participation of either receptors in the regulation of the gene encoding *CYP27A* still remains to be demonstrated. Indeed, to date the mode of action, most particularly at the nuclear level, of the putative VDR_m still remains to be demonstrated while the *CYP27A* gene promotor has not been shown to harbor the VDRE DR3 consensus sequence ([G/A]GGT[G/C]A) (21;56). Interestingly, however, $1,25(OH)_2D_3$ has lately been shown to influence the expression of the gene encoding *CYP3A4* by a mechanism which did not involve a direct VDR_n-RXR-VDRE interaction but involved rather a VDR_n-RXR-PXRE interaction through an ER6 motif indicating that $1,25(OH)_2D_3$-signaling can also be mediated through complexed nuclear cross talk with several response motifs (61).

The long *CYP27A* mRNA half-life as well as the slight, albeit not significant, $1,25(OH)_2D_3$-mediated increase in its mRNA half-life in the presence of a significant inhibition in the expression of the gene transcript may explain the rather slow and progressive decrease in *CYP27A* mRNA levels observed over the 7 day period studied. The later observations, combined with the known long (several weeks) serum $25OHD_3$ half-life, suggest that exogenously administered $1,25(OH)_2D_3$ would most likely not translate, in the short run, into a functional D deficiency *in vivo*. In addition, it is not excluded that in the presence of low *CYP27A* levels, other cytochrome(s) P-450 could contribute to the production of the prohormone. Indeed, depending on the experimental design and substrate doses used, data on the effect of D_3 or D_3 metabolites on the production of $25OHD_3$ have led to conflicting results with evidence of regulation (10;12;41), absence of regulation (19;33;46;63), or regulation not directly involving the enzyme but other mechanisms such the metabolic or biliary clearances of the vitamin

and/or its metabolites (9;18;31;32). Moreover, patients suffering form cerebrotendinous xanthomatosis (CTX) have also been reported to have a wide range of circulating $25OHD_3$ concentrations (from very low to near normal) (22) indicating that enzyme(s) other than *CYP27A* can transfrom D_3 into $25OHD_3$ as illustrated in a *CYP27A*-ablated mouse model (47).

Exogenously administered low dose D_3 (which led, however, to the normalization of serum Ca^{2+} and the secondary hyperparathroidism (data not shown)) or $25OHD_3$ administration were found not to significantly affect *CYP27A* mRNA. A higher dose of D_3 only led to a transient decrease in *CYP27A* which promptly returned to D depleted values following one week of repletion despite elevated serum Ca^{2+} concentrations. The data indicate that the D_3 nutritional status has no significant effect on the total hepatic *CYP27A*. Paradoxically, evaluation of the D_3 encodrine status revealed that the circulating $1,25(OH)_2D_3$ concentrations and circulating Ca^{2+} were significantly higher in D_3-repleted animals compared to $1,25(OH)_2D_3$-repleted animals. This observation indicates that the circulating $1,25(OH)_2D_3$ concentration is not a good predictor of the hepatic action of the hormone and that the protective effect against the down-regulatory effect on *CYP27A* may rest on the circulating or cellular levels of D_3 or $25OHD_3$. It is also postulated that D_3 or $25OHD_3$ may compete with $1,25(OH)_2D_3$ for uptake by the liver. In fact, the season of the year (summer/fall), or the circulating concentrations of $25OHD_3$ and the hepatic mRNA levels of *CYP27A* have already been reported in human subjects while no correlation was observed between the circulating $1,25(OH)_2D_3$ concentration and the *CYP27A* gene transcript (23).

Interestingly, we recently reported a negative regulation of the intestinal *CYP27A*

following repletion with D_3, $25OHD_3$ as well as $1,25(OH)_2D_3$ (59) indicating differences between the two organs in the overall regulation of *CYP27A* by the D_3 nutritional/endocrine system. Moreover, the sensitivity of the hepatic *CYP27A* to $1,25(OH)_2D_3$ was also shown to be higher (with a 60% decrease *CYP27A* mRNA) than that found in the intestine (40% decrease). The reasons for the observed differences between the two organs in the regulation of the gene encoding *CYP27A* by the nutritional status may rest in the fact that the intestine, but not the liver, harbors the 1α-hydroxylase allowing intestinal cells to locally produce $1,25(OH)_2D_3$ following D_3 or $25OHD_3$ administration. On the other hand, the influence of $1,25(OH)_2D_3$ on the *CYP27A* mRNA half-life as well as on the *CYP27A* transcription rate was also shown to be much more pronounced in liver than in intestine. It is postulated that the high sensitivity of the liver to $1,25(OH)_2D_3$ administration may be due to a combination of factors such as the efficient hepatic capture of exogenously administered $1,25(OH)_2D_3$ (27) combined with the absence of the D_3-24 hydroxylase which is, however, present in intestine (20;49) and which could contribute to the intestinal catabolism of exogenously administered $1,25(OH)_2D_3$, as well as in differences in cellular life span with hepatocytes having a life span of several months while the turnover of intestinal cells is only a matter of days.

The present studies thus clearly show that in rodent liver, the gene encoding *CYP27A* is expressed not only in hepatocytes but also in all sinusoidal cells. They also illustrate a significant gender difference in steady-state *CYP27A* mRNA levels, and show that $1,25(OH)_2D_3$ administration significantly influence the transcription of the *CYP27A* gene which was accompanied by a significant decrease in the mitochondrial C-25 hydroxylation of the model D_3 compound $1\alpha OHD_3$. Our data also illustrate that

$1,25(OH)_2D_3$ stands to also affect the synthesis of bile acids via a down-regulation of the secondary "acidic" pathway.

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TABLE 1

Serum vitamin D metabolites and Ca^{2+} concentrations

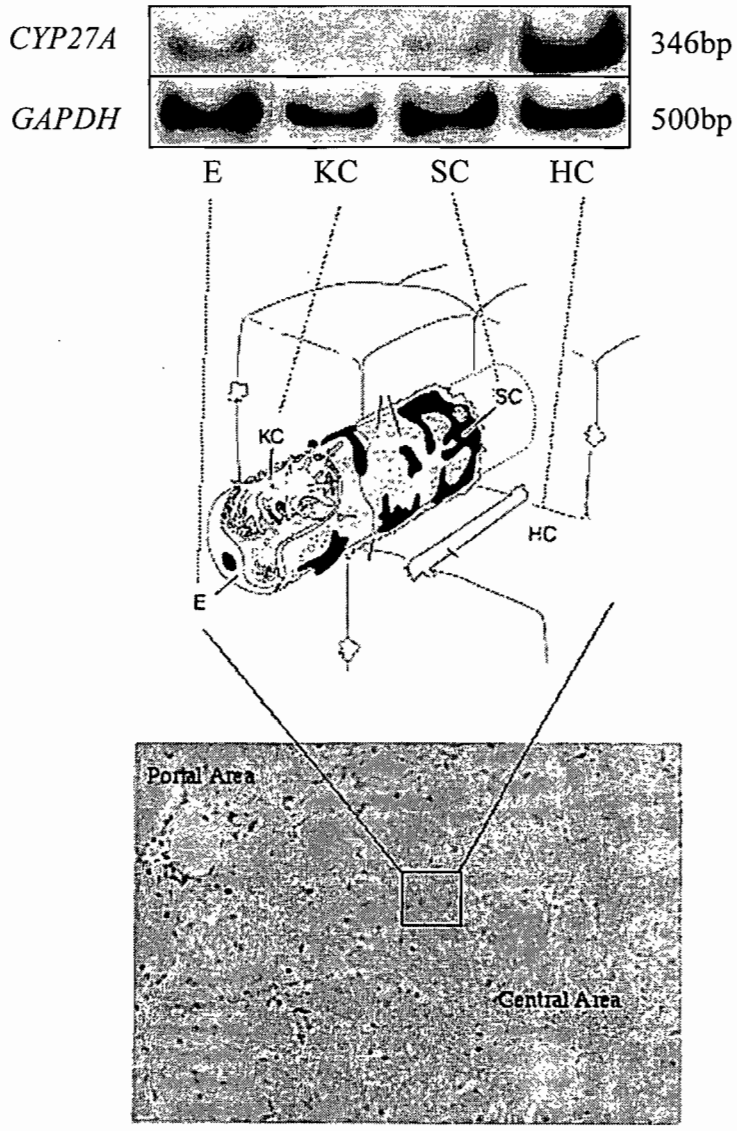
Groups	$25OHD_3$ (mmol/L)	$1,25(OH)_2D_3$ (pmol/L)	Ca^{2+} (mmol/L)
D-Ca-	9 ± 0.8	28 ± 2	$0.85 \pm 0.006^{***}$
D_3 (6.5 mmol/d)	$28 \pm 3^{*}$	$1019 \pm 77^{**}$	$1.24 \pm 0.003^{**}$
D_3 (32.5 mmol/d)	$75 \pm 12^{*}$	$1308 \pm 130^{**}$	$1.45 \pm 0.05^{***}$
$25OHD_3$ (28 pmol/d)	3 ± 2	533 ± 91	1.13 ± 0.04
$1,25(OH)_2D_3$ (28 pmol/d)	9 ± 4	565 ± 132	1.11 ± 0.04

Serum $25OHD_3$ and $1,25(OH)_2D_3$ concentrations following 7 days of repletion with D_3, $25OHD_3$ or $1,25(OH)_2D_3$ administrated i.p. by osmotic mini-pumps. Data are presented as means ± S.E.M. n = 3 rats/group. Statistically significant differences between group means were evaluated by the Student's "t" test in relation the values obtained in $1,25(OH)_2D_3$ repleted animals. * $p<0.0001$, ** $p<0.05$, *** $p<0.005$.

LEGEND TO FIGURE 1

Steady state expression of the gene encoding the *CYP27A* gene transcript in freshly isolated hepatocytes and in hepatic sinusoidal cells obtained from D depleted male rats. Representative RT-PCR of the *CYP27A* gene transcript in hepatocytes (HC), stellate cells (SC), endothelial cells (E) and Kupffer cells (KC). The illustration is a modification based on McCuskey (39).

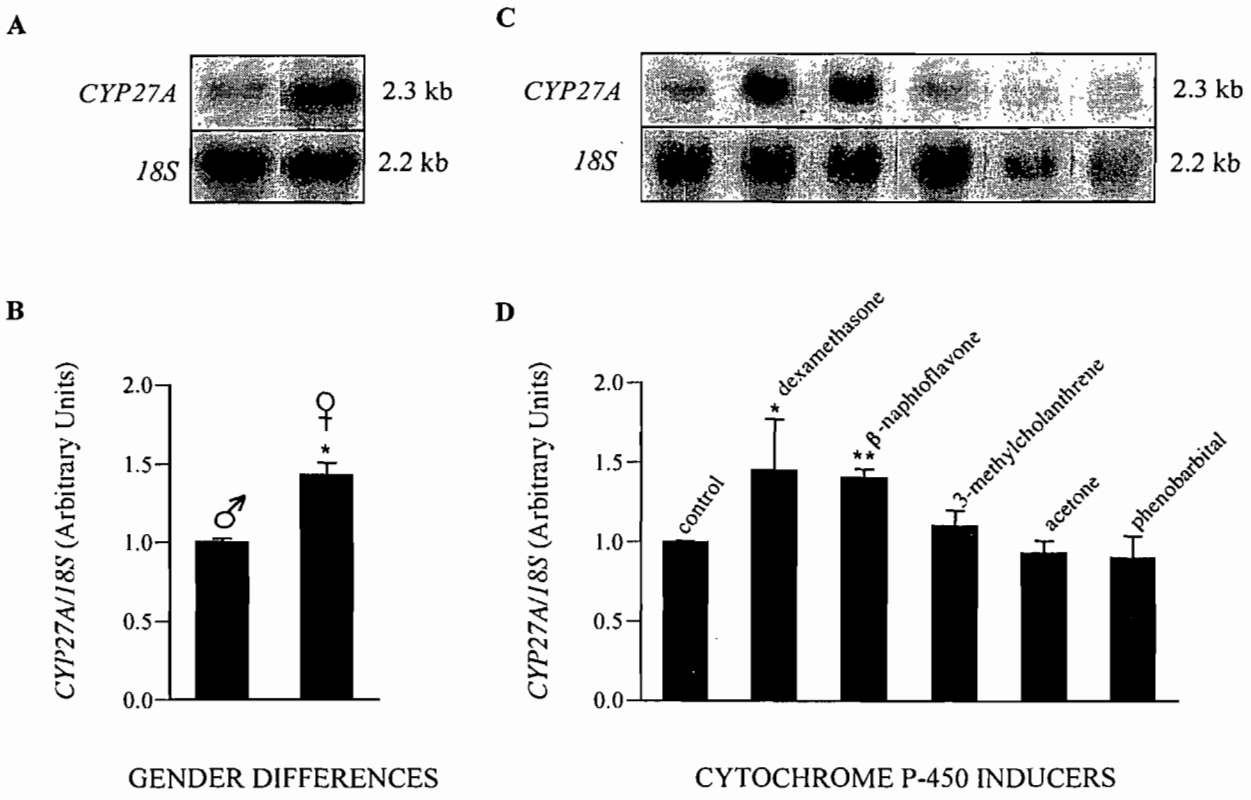
Figure 1



LEGEND TO FIGURE 2

Influence of gender and cytochrome P-450 inducers on the steady state expression of *CYP27A* mRNA level. Representative Northern blot analyses of of the *CYP27A* gene transcript in male and female rat livers (A), and following exposure to cytochrome P450 inducers (C). B. and D. Means ± S.E.M. of *CYP27*A/18S mRNA ratio assed by densitometric scanning. n = 4-5 animals/group for the studies on gender differences and n = 3 animals/group for the studies on cytochrome P-450 inducers. Statistically significant differences were evaluated by the Student's "t" test, * $p<0.05$, ** $p<0.0003$.

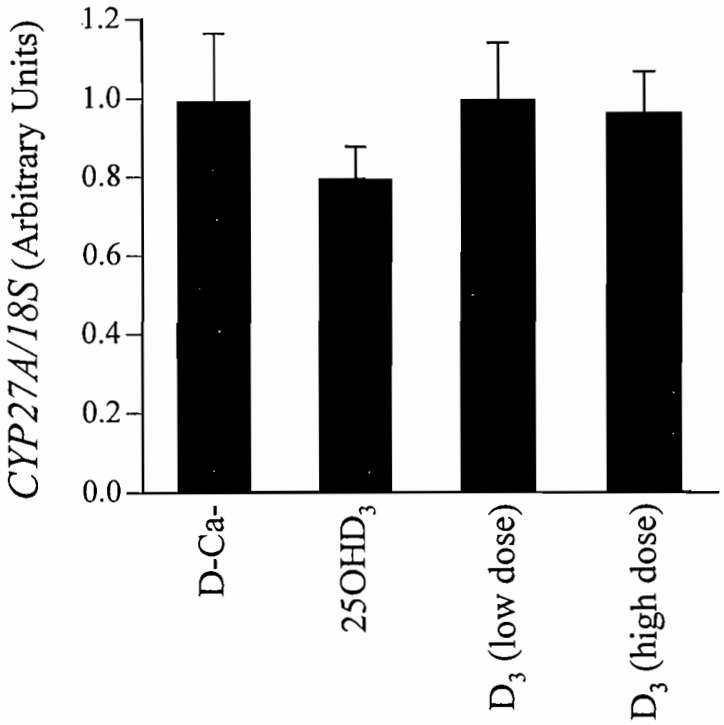
Figure 2


A
CYP27A
18S
2.3 kb
2.2 kb
C
CYP27A
18S
2.3 kb
2.2 kb
B
CYP27A/18S (Arbitrary Units)
2.0
1.5
1.0
0.5
0.0
♂
♀
*
GENDER DIFFERENCES
D
CYP27A/18S (Arbitrary Units)
2.0
1.5
1.0
0.5
0.0
control
* dexamethasone
** β-naphtoflavone
3-methylcholanthrene
acetone
phenobarbital
CYTOCHROME P-450 INDUCERS

LEGEND TO FIGURE 3

Influence of D_3 and $25OHD_3$ repletion of the hepatic expression of the gene encoding *CYP27A*. A. Representative Northern analyses of the *CYP27A* steady-state *CYP27A* mRNA levels in D-depleted, and in D_3 (6.5 nmol/day (low dose) or 32.5 (high dose) nmol/day), or $25OHD_3$-repleted (6.5 nmol/day) rats following 7 days of repletion by osmotic mini-pump (i.p.). B. Densitometric analyses of the data obtained are presented as Means ± S.E.M. D-depleted rats, n = 15, D_3 and $25OHD_3$ n = 3 animals/group. Statistically significant differences between group means were analysed ANOVA with individual contrasts evaluated by the Bonferronni *post hoc* test.

Figure 3

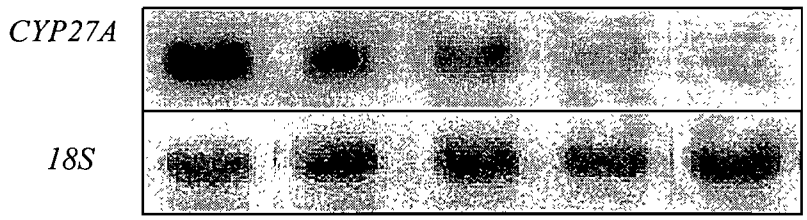

CYP27A/18S (Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
D-Ca-
25OHD3
D3 (low dose)
D3 (high dose)

LEGEND TO FIGURE 4

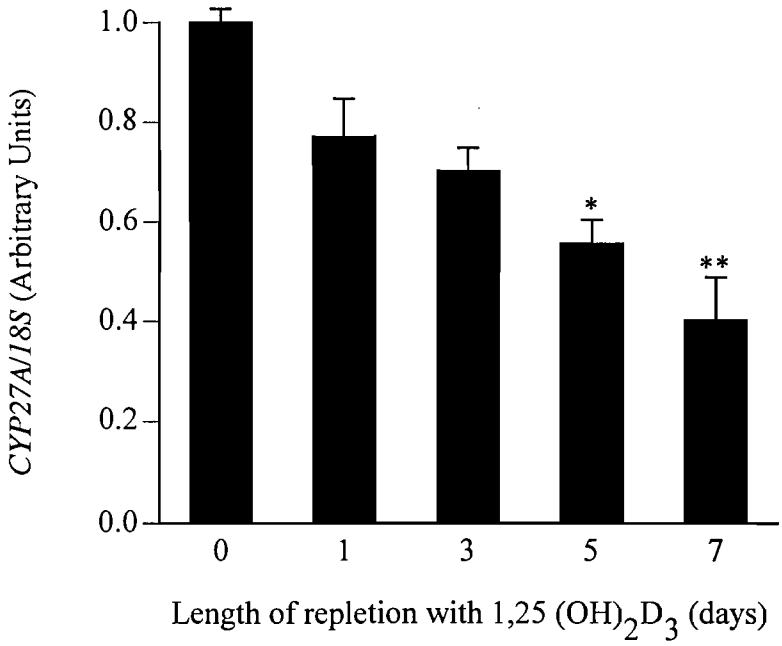
Steady-state levels of hepatic *CYP27A* mRNA in D-depleted rats repleted with $1,25(OH)_2D_3$ rats following 1 to 7 days of repletion. A. Representative Northern blot analyses of the *CYP27A* gene transcript. B. Densitometric analyses. Data are presented as means ± S. E. M. Statistically significant differences between group means were analysed by ANOVA with individual contrasts evaluated by the Bonferonni *post hoc* test. Main effect $p<0.0001$. Statistically significantly different from D-Ca-, *$p<0.001$, **$p<0.0001$. n = 3 animals/group.

Figure 4

A


CYP27A
18S

B


CYP27A/18S (Arbitrary Units)
1.0
0.8
0.6
0.4
0.2
0.0
*
**
0
1
3
5
7
Length of repletion with 1,25 (OH)2D3 (days)

LEGEND TO FIGURE 5

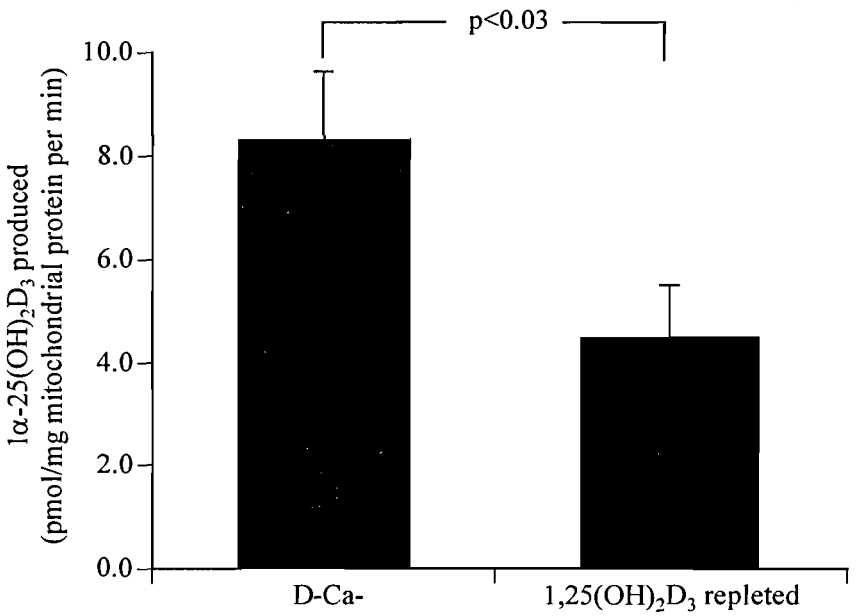
Representative photomicrographs of rat liver sections obtained following *in situ* hybridization using a *rCYP27A* antisens riboprobe. A 10 RT-PCR cycle amplification was used. Liver sections were obtained from D depleted rats (A, B), normal control rats (D, E) and 7 day $1,25(OH)_2D_3$ repleted rats (G, H). Negative *in situ* hybridization control using the *rCYP27A* sense ribobrobe are presented in panels C, F, and I for livers obtained from D depleted, normal and $1,25(OH)_2D_3$ repleted rats respectively.

Figure 5

LEGEND TO FIGURE 6

Effect of $1,25(OH)_2D_3$ on the hepatic mitochondrial C-25 hydroxylation activity. Hepatic mitochondrial proteins from hypocalcemic D depleted and $1,25(OH)_2D_3$-repleteded (28 pmol/d for 7 days) were incubated with 20 nmol of $1\alpha OHD_3$. The product of the reaction $1\alpha,25(OH)_2D_3$ was evaluated by HPLC. Data are presented as means ± S.E.M. Statistically significant differences between group means were analysed by the Student's "t" test, n = 8-11 animals/group, $p<0.03$.

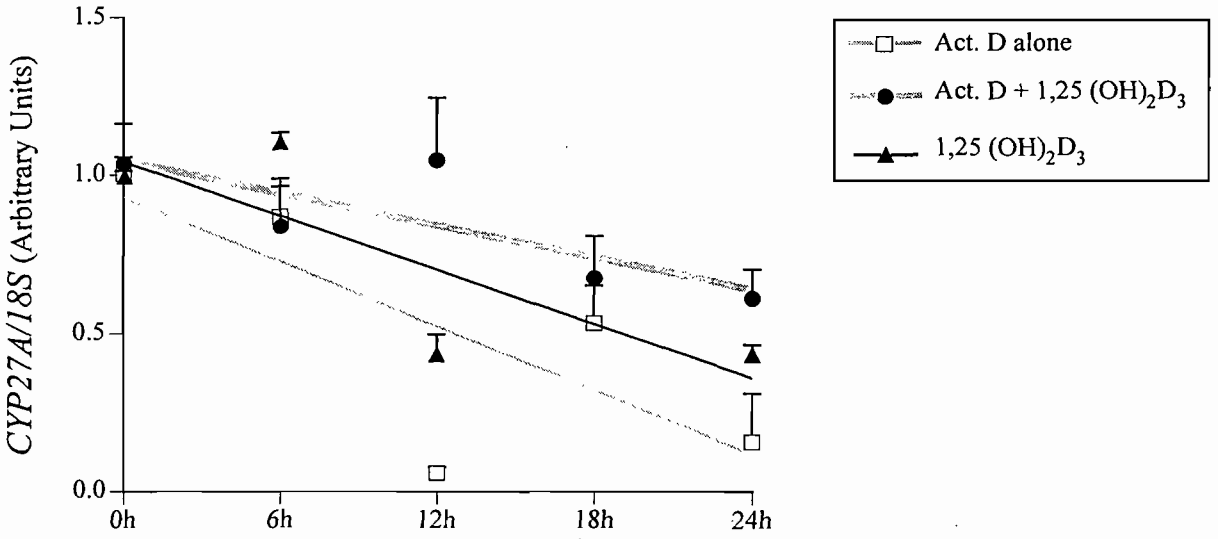
Figure 6


p<0.03
10.0
8.0
6.0
4.0
2.0
0.0
1α-25(OH)2D3 produced
(pmol/mg mitochondrial protein per min)
D-Ca-
1,25(OH)2D3 repleted

LEGEND TO FIGURE 7

In vivo influence of $1,25(OH)_2D_3$ on the half-life of *CYP27A* mRNA. Animals received i.p. doses of actinomycin D (0.5mg/kg) every 6 hours and were kept either untreated or received 12 nmol/kg i.v. dose $1,25(OH)_2D_3$ every 6 hours. Animals were killed 6, 12, 18 and 24 hours after actinomycin D administration. Data are presented as means ± S. E. M. n = 4-5 animals/group. Statistically significant differences between group means were analysed by AVOVA with individual contrasts evaluated by the Bonferonni *post hoc* test. No significant differences were observed between the different groups.

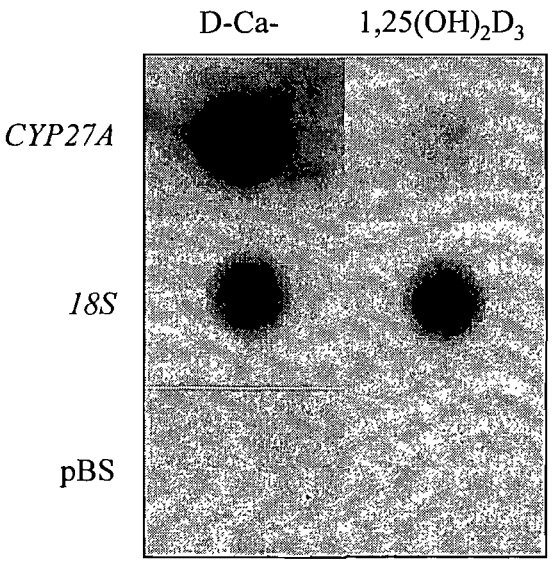
Figure 7


1.5
1.0
0.5
0.0
CYP27A/18S (Arbitrary Units)
0h
6h
12h
18h
24h
Act. D alone
Act. D + 1,25 (OH)2D3
1,25 (OH)2D3

LEGEND TO FIGURE 8

Rate of transcription of the *CYP27A* gene transcript in livers obtained from D depleted and $1,25(OH)_2D_3$ injected rats. Animals received a single i.v. dose (12nmol/kg) of $1,25(OH)_2D_3$ 6 hours before euthanasia. Transcriptional activity was measured in quadruplicate using 2-3 rat livers/group. The illustration presents a representative nuclear run-on transcriptional assay.

Figure 8



2.2.2 Article 2: Expression of CYP27A, a gene encoding a vitamin D-25 hydroxylase in human liver and kidney.

Article published in the Journal of Clinical Endocrinology, volume 54, pages 107-115 (2001).

(ARTICLE 2)

EXPRESSION OF *CYP27A*, A GENE ENCODING A VITAMIN D-25 HYDROXYLASE IN HUMAN LIVER AND KIDNEY

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Key words: hCYP27A, cytochrome P-450, D_3 25-hydroxylase, 25OHD_3, vitamin D, liver, kidney

Short title: Human vitamin D-25 hydroxylase

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SUMMARY

OBJECTIVES Vitamin D_3 (D_3) is not active but must be hydroxylated at C-25 in liver before acquiring its hormonal potential in the kidney. The sterol-27 hydroxylase (gene symbol: *CYP27A*) catalyses the oxidation of sterol side chain in bile acid synthesis but the enzyme is also known as a D_3-25 hydroxylase. *Aims:* The study examined the expression of the gene encoding *CYP27A* in adult and fetal human livers and kidneys. SUBJECTS 39 adults (18 men and 21 women) (mean age 58 years in men and 57 years in women) and 3 normal fetuses gestational age 17-19 weeks were studied. RESULTS *I) Normal specimens: CYP27A* transcript was found to be higher in adult than in fetal livers but its expression was similar in adult and fetal kidneys. In fetuses, no difference was observed between *CYP27A* levels in livers and kidneys. In adult livers *CYP27A* levels were higher in women than in men. Hepatic *CYP27A* mRNA and serum 25OHD concentrations were both found to be higher in summer than in winter. Multiple correlation analyses indicate that the season of the year and the serum 25OHD concentrations (but not $1,25(OH)_2D$ concentrations) are the best predictors of *CYP27A* mRNA abundance in normal adult livers. *In situ* hybridization illustrates a clear label in hepatocytes which increases in intensity in the perivenous region of the hepatic acinus. *II) Pathological specimens:* In one man, an hepatic carcinoma exhibited a very large increase in *CYP27A* (>1000 fold) compared to the level found in the normal area. In that patient, serum 25OHD concentrations were found to be high considering the level of *CYP27A* mRNA in the normal hepatic area suggesting that the neoplastic tissue contributed to the C-25 hydroxylation of vitamin D. Specimens obtained from two patients suffering from focal hepatic hyperplasia indicate that in one case, the level of *CYP27A* mRNA was twice as high in the pathological than in the normal area while in the other its levels were similar in both areas. No difference in the *CYP27A* transcript was observed between specimens obtained from normal areas and those obtained form either an hepatic adenoma or from two intrahepatic colon metastases. CONCLUSION *CYP27A* is present not only in the human adult liver but also in the adult kidney, and in the fetal liver and kidney. The findings illustrate that *CYP27A* can be significantly upregulated in certain pathological situation such as in hepatic carcinoma and that the neoplastic tissue could contribute to the circulating concentration of 25OHD.

INTRODUCTION

Vitamin D_3 (D_3) is an endogenous secosteroid synthesized in the skin under the ultraviolet rays of the sun. D_3 is not active but must be hydroxylated at position C-25 in the liver before acquiring its full hormonal potential through a C-1α hydroxylation in kidney mitochondria (Gascon-Barré, 1997). Animal studies have identified two intra-hepatic organelles, the smooth endoplasmic reticulum and the mitochondrium as sites possessing fully active but distinct D_3-25 hydroxylases (Gascon-Barré, 1997). The mitochondrial enzyme has been identified in a small number of human livers (Bjorkhem *et al.* 1975; Bjorkhem & Holmberg, 1978; Oftebro *et al.* 1981) and claimed to be the unique site for the C-25 hydroxylation of D_3 in humans (Saarem *et al.* 1984; Saarem & Pedersen, 1985).

The mitochondrial D_3-25 hydroxylase was first identified as the C-27 sterol hydroxylase which catalyses the first and rate-limiting step in the oxidation of the sterol side chain in the «acidic» bile acid biosynthesis pathway (Bjorkhem, 1985). Cali and Russell (Cali & Russell, 1991) termed the enzyme sterol-27 hydroxylase although the enzyme exhibits low substrate specificity. Cloning of the rabbit (Andersson *et al.* 1989), rat (Usui *et al.* 1990; Su *et al.* 1990), and human (Cali & Russell, 1991; Guo *et al.* 1993) sterol-27 hydroxylase was achieved between 1989 and 1993 and the gene encoding the enzyme has been termed *CYP27A*. A porcine microsomal vitamin D 25-hydroxylase (*CYP25*) exhibiting an 86% homology with the human *CYP2D6* has been cloned by Wikvall and his group (Postlind *et al.* 1997) but Gill *et al.* (Gill *et al.* 2000) have lately orally reported that the porcine *CYP25* could not be found in any other species.

Evaluation of D_3-25 hydroxylase activity revealed that $25OHD_3$ production was present not only in livers but also in extra-hepatic tissues (Tucker, III. *et al.* 1973; Bhattacharyya & DeLuca, 1974). Support for the extra-hepatic presence of a D_3-25 hydroxylase first came from *in vivo* studies reporting that hepatectomy markedly reduced but did not eliminate the plasma appearance of $25OH[^3H]D_3$ after $[^3H]D_3$ injection (Ponchon *et al.* 1969; Olson *et al.* 1976). These observations have now been confirmed at the molecular level with demonstration of the presence of *CYP27A* mRNAs in several tissues and organs (Cali & Russell, 1991; Ichikawa *et al.* 1995; Andersson *et al.* 1989; Mullick *et al.* 1995). The participation of the extra-hepatic enzyme to the production of $25OHD_3$ or its contribution to

the circulating concentration of $25OHD_3$ under normal physiological conditions is not, however, presently known. Moreover, Axén *et al.* (Axén *et al.* 1994) have reported that the sterol-27 hydroxylase purified from pig and rabbit livers as well as recombinant human *CYP27A* expressed in *Escherichia coli* or monkey COS cells was also able to catalyse the 1α-hydroxylation of $25OHD_3$ albeit at a much lower rate than that observed for the conversion of D_3 into $25OHD_3$ (Axén *et al.* 1995) and at several order of magnitude lower than the $25OHD_3$-1α hydroxylase (CYP27B1) (Pikuleva *et al.* 1997).

The aim of the present studies was to examine the steady-state expression of the gene encoding *CYP27A* in the adult and fetal human livers and kidneys and, in adult volunteers, to evaluate its level of expression in relation to the vitamin D status as well as to compare its level of expression in normal and pathological hepatic specimens in a subset of patients.

MATERIALS AND METHODS

CHEMICALS

pBluescript SK (-) vector used for Northern analyses was obtained from Stratagen, LaJolla, CA, USA. William's medium E and Trizol LS reagents, yeast tRNA were from Gibco BRL (Burlington, ON, Canada). Hep G2 and Hep 3B cell lines were obtained from American Type Culture Collection (Rockville, MD). Klenow fragment, T7 RNA polymerase and dextran sulfate were from Amersham Pharmacia Biotech (Baie D'Urfé, Qc, Canada), proteinase K, ribonuclease A and salmon sperm DNA from Sigma Chemical Co. (Mississauga, ON, Canada), OneStep RT-PCR from Qiagen (Mississauga, ON, Canada), [α-^{32}P] dCTP and [α-^{33}P] UTP from ICN Biomedicals Inc (Mississauga, ON, Canada), Kodak NBT-2, D19 developer and fixer from Interscience (Mississauga, ON, Canada). 1,25$(OH)_2D_3$ was a gift from Hoffmann-LaRoche Company (Nutley, NJ,USA). All other materials were of analytical grade or better.

SUBJECTS CHARACTERISTICS

Protocols were approved by the Institutional Ethics Review Board and informed consents were obtained from all subjects. Adult subjects were chosen from patients referred for hepatic or renal surgery while normal fetal tissues were obtained following voluntary termination of pregnancy. Description of the population studied is presented in Figure 1. As indicated, 39 adults (18 men, mean age: 58.1 years ± 2.0, and 21 women, mean age: 57.1 ± 3.7 years) and 3 normal fetuses gestational age 17 to 19 weeks were included in the study. In adults, specimens were obtained form normal and, when possible, from pathological areas of the liver as well as from normal kidney tissue. In fetuses, normal liver and kidney specimens were obtained.

LABORATORY METHODS

Evaluation of the CYP27A gene transcript

Total liver or renal RNA (15 µg) was blotted onto membrane and processed for Northern analyses as described earlier (Demers *et al.* 1997). The radiolabeled probes were: *CYP27A*, a 2.1 kb human cDNA insert from the EcoRI site of the pBluescript SK (-) vector;

ribosomal *18S* RNA a 1.5 kb human cDNA insert from the EcoRI site of the pBluescript SK (-) vector (ATCC (#77242). The probes were labelled by random oligo-priming (1913) using [α-32P] dCTP (3000 Ci/mol) and Klenow fragment. Blot hybridization, washing, exposure, and photodensitometric evaluation were performed as described previously (Demers *et al.* 1997).

Normal human hepatocytes were isolated by the method of Guguen-Guillozo *et al.* (Guguen-Guillouzo *et al.* 1982), and the freshly isolated hepatocytes were equilibrated in William's E medium. Total RNA from normal human hepatocytes and from the hepatocellular cell lines Hep G2 and Hep 3B were extracted by Trizol LS reagent and used for Northern analyses as mentioned above.

In situ RT-PCR hybridization

Paraffin liver sections were mounted onto slides pre-treated with APES, dewaxed in xylene and then rehydrated trough a series of ethanol baths, and finally immersed in DEPC-treated water. Slides were incubated for 30 min. with 100μL 0.1 M Tris-HCl pH 8.0, 50 mM EDTA and 50μg/mL proteinase K, fixed in fresh 4% paraformaldehyde-PBS solution for 20 min., rinsed in PBS and then rinsed in 90% ethanol and allowed to dry. Negative control samples were obtained by treatment of sections with 100μg/mL RNase A in 2 x SSC at 37°C for 30 min. and washed in PBS prior to RT-PCR reaction. All other treatments were the same.

In situ RT-PCR were performed by the method of Mee et al. (Mee *et al.* 1996) with the following modifications. Reaction was carried out in a volume of 50μL with OneStep RT-PCR kit and 0.6μM hCYP27A specific primers based on the published sequence of Guo et al. (Guo *et al.* 1991) using a Hybaid thermal cycler provided with a *in situ* block (Hybaid, Teddington, UK). Sections were heated for 30 min. at 50°C, 15 min. at 95°C and 10 cycles of 95°C for 30 sec., 62°C for 1min. and 72°C for 1 min. were performed, finally slides were heated for 10 min. at 72°C. Sample were washed twice in PBS and fixed in 4% Paraformaldehyde-PBS for 20 min. at 4°C and incubated with 0.25% acetic anhydride in 0.1 TEA for 10 min. and rinsed in 90% ethanol and allowed to dry.

An *in situ* hybridization was performed using a previously described method (Roy & Tenenhouse, 1996) with some modifications. The *hCYP27A* anti-sense riboprobes (using linearised *CYP27A* cDNA as template) were generated by the single strand RNA synthesis

technique using T7 RNA polymerases and [α-^{33}P]UTP (800 Ci/mmol). Hybridization was performed at 42°C for 16 hours with 100µL of hybridization solution (50% formamide, 2XSSC, 1X Denhart's, 0.25M Tris-HCl, pH 7.5, 10% dextran sulfate, 0.5 Na pyrophosphate, 0.5% SDS, 25µg/mL denatured salmon sperm DNA, 250 µg/mL yeast tRNA) and 1 X10^7 cpm/mL of antisense. After washing, autoradiography was performed with NBT-2 emulsion. Slides were exposed for 7 days at 4°C, developed with D19 developer and counterstained with heamatoxylin and eosin.

Biochemical analyses

Concentrations of ionized calcium in whole blood were measured with a ICA2 ionized calcium analyser (Radiometer, Copenhagen, Denmark). Total calcium, phosphate, bilirubin, AST, ALT, albumin, and alkaline phosphatase were determined by automated colorimetry (Baxter, Paramax, Irvine, CA, USA) by the Clinical Biochemistry Department of the CHUM St-Luc Hospital. Serum D metabolites 25(OH)D and $1,25(OH)_2D$ were measured using the Incstar 25(OH)D and $1,25(OH)_2D$ RIA kits (Incstar Corporation, Stillwater, MN) containing a radio-iodinated 25(OH)D and $1,25(OH)_2D$ analog respectively.

STATISTICAL ANALYSIS

Results are presented as means ± SEM. Statistically significant differences between group means were evaluated by analysis of variance or the Student's «t» test as indicated in the Figure legends.

RESULTS

Values for the serum biochemical analyses obtained from adult volunteers are presented in Table 1. As indicated, most biochemical parameters studied were within the normal range except for the ionized calcium and albumin concentrations which were found to be lower than normal, and the concentrations of AST and ALT in women and alkaline phosphatase in men which were found to be higher than normal. In all cases, however, no significant differences between the values obtained in men and women were observed.

Figure 2 presents the level of the *CYP27A* gene transcript in normal specimens of adult and fetal livers and kidneys. As illustrated, the level of the hepatic *CYP27A* transcript was found to be higher in specimens obtained from adult than in those obtained from fetal livers ($p<0.05$). *CYP27A* was detected in kidney of both adult and fetal specimens but no significant differences in the steady state level of the gene transcript were observed between adult and fetal kidney specimens. The level of the *CYP27A* gene transcript was found to be significantly higher in adult liver than in adult ($p<0.0001$) or fetal kidney ($p<0.01$). In fetal specimens, no significant difference was observed in the level of *CYP27A* mRNA between liver and kidney specimens.

In situ hybridization of normal liver specimens illustrates that *CYP27A* is expressed in hepatocytes throughout the hepatic acinus (Fig. 3). However, an intensity gradient from the portal area to the perivenous region of the hepatic acinus is apparent suggesting an intrahepatic regionalisation of *CYP27A* in the human liver.

The comparative steady state expression of *CYP27A* in normal specimens obtained from liver and kidney of men and women is presented in Figure 4. The levels of the *CYP27A* transcript were found to be slightly higher in specimens obtained from women than in those obtained from men ($p<0.05$) but no significant difference between men and women was observed in the level of the kidney transcript. As indicated in Table 1, circulating 25OHD concentrations were found to be similar in men and women (40.8 ± 6.2 and 45.5 ± 7.2 nmol/L in men and women respectively).

As illustrated in Figure 5, significant differences in the hepatic level of *CYP27A* mRNA (Fig. 5A) and in the serum 25OHD (Fig. 5B) concentrations were observed between specimens obtained in spring/summer and those obtained in fall/winter with significantly

higher values observed for both parameters in spring/summer than in fall/winter ($p<0.05$). A multiple correlation analysis indicated that the season of the year ($p<0.01$) and the circulating 25OHD concentrations ($p<0.05$) are the best predictors of the mRNA abundance for the gene encoding *CYP27A* in normal adult livers. As shown in Figure 5C, this observation is illustrated by a significant linear correlation between the circulating 25OHD concentrations and the hepatic level of *CYP27A* mRNA. No significant correlation was found between the hepatic level of *CYP27A* mRNA levels and the circulating albumin ($p=0.47$) or $1,25(OH)_2D$ ($p=0.66$) concentrations. In kidney, no significant correlation was observed between serum 25OHD or $1,25(OH)_2D$ concentrations and the levels of *CYP27A* mRNA (data not shown).

Figure 6 illustrates the hepatic *CYP27A* steady-state expression in normal and pathological specimens obtained from six men and women. A very large difference in the level of the *CYP27A* transcript was observed between the specimen obtained from the normal hepatic area and that obtained from an hepatic carcinoma area in one man. In that patient, the serum 25OHD concentrations were found to be high considering the level of *CYP27A* mRNA in the normal hepatic area suggesting that the neoplastic tissue contributed to the C-25 hydroxylation of the vitamin. Evaluation of *CYP27A* mRNA level in the hepatocellular carcinoma cell line HepG2 and in the hepatoblastoma cell line Hep3B did not suggest, however, an increased expression when compared to that observed in primary culture of normal human hepatocytes (Fig. 6B). Specimens were also obtained from two patients suffering from focal hepatic hyperplasia. In one woman, the level of *CYP27A* mRNA was found to be twice as high in the pathological than in the normal area while in the other the steady-state expression of *CYP27A* was found to be similar in both areas. No difference in the level of the *CYP27A* transcript was observed between the specimens obtained form normal areas and those obtained form either an hepatic adenoma or from two intrahepatic colon metastases.

DISCUSSION

The data obtained during the present studies clearly demonstrate that *CYP27A* is expressed in the adult and fetal liver and kidney. *In situ* hybridization of normal adult livers illustrates that the gene encoding *CYP27A* is well expressed throughout the hepatic acinus but that its intensity increases in the perivenous area indicating that a predominant perivenous regionalisation of the gene is present in humans. In the rat (Wiese *et al.* 1992; Vlahcevic *et al.* 1996) but not the rabbit (Araya *et al.* 1995), *CYP27A* is highly sensitive to the prevailing concentrations of bile acids with increases in enzyme activity, steady state mRNA level, and the rate of gene transcription following interruption of the enterohepatic circulation of bile acids (Vlahcevic *et al.* 1996; Twisk *et al.* 1995b). Moreover, a regio-selective dynamic response of the liver CYP27A following depletion of the bile acid pool has been reported in the rat with significant increases in the perivenous but not in the periportal area of the acinus (Twisk *et al.* 1995b). The volunteers who participated in the studies were not reported to present significant cholestasis suggesting that in the normal human liver the mitochondrial D_3-25 hydroxylase seems to be predominantly located in the distal area of the hepatic acinus.

Our study shows that the liver of 17 to 19 week old human fetuses express the *CYP27A* gene transcript albeit at a much lower level than that observed in the adult liver. This observation is congruent with observations that 25OHD is lower in cord than in maternal serum although, a significant relationship is observed between the two sites (Delvin *et al.* 1982). It is also congruent with data showing that premature infants have low circulating 25OHD concentrations than infants born at term (Hillman *et al.* 1985; Salle *et al.* 1983).

Although, in rat hepatocytes, the *CYP27A* mRNA half-life has been reported to be between 18 and 24 hours suggesting a slow response to regulators (Stravitz *et al.* 1996), several hormones and endogenous products have already been shown to regulate the gene. They include, as reported above, bile acids but also growth hormone, glucocorticoids, insulin and the physiological state of the animal (Su *et al.* 1990; Mullick *et al.* 1995; Vlahcevic *et al.* 1996; Twisk *et al.* 1995a; Twisk *et al.* 1995b). To date, however, most regulation studies on *CYP27A* have been linked to cholesterol and/or bile acids metabolism and few studies

have addressed the effect of these hormones on the handling of vitamin D. The present study points to a possible regulation by the vitamin D status with higher level of *CYP27A* expression being observed when the circulating 25OHD concentrations are high than when they are low, although the present study does not allow to draw conclusions on a causal relationship between the two parameters. Interestingly, however, Lehmann *et al.* (Lehmann *et al.* 1997) have lately reported that in human keratinocytes, *CYP27A* is inducible by D_3 or UVB irradiation suggesting the presence of a positive regulation of the vitamin (or of its products) on the mitochondrial D_3-25 hydroxylase in these cells.

The regulation of the human enzyme by sex hormones has not been studied. The mitochondrial enzyme has been shown in the rat to be several times more active in females than in males (Saarem & Pedersen, 1987; Addya *et al.* 1991). In addition, Saarem and Pedersen (Saarem & Pedersen, 1987) observed that enzyme activity increased when β-estradiol was injected to male while testosterone decreased enzyme activity when injected to female rats. Addya *et al.* (Addya *et al.* 1991) also reported that the relative level of the D_3-25 hydroxylase was reduced in castrated females while it was increased in castrated males. Our studies indicate that in the human liver, the steady-state level of *CYP27A* mRNA is higher in women than in men despite the subjects' age (average age in women 57 years indicating a post-menopausal status in most subjects).

The sterol-27 hydroxylase has been claimed to be the only enzyme involved in the C-25 hydroxylation of D_3 in humans. However, mutations affecting either *CYP27A* expression or its primary sequence (leading to cerebrotendinous xanthomathosis, an inherited disorder of sterol metabolism and storage characterized by atherosclerosis and progressive neurological dysfunction) does not seem to completely abolish the C-25 hydroxylation of D_3 (Kuriyama *et al.* 1993; Leitersdorf *et al.* 1993; Berginer *et al.* 1993; Leitersdorf *et al.* 1994). This observation suggests the presence of alternative, but as yet, unidentified D_3-25 hydroxylase(s) in humans. In addition, the human cell line Hep3B has also been shown to efficiently activate the synthetic compound 1α-hydroxyvitamin D_3 at C-25 despite the absence of any detectable *CYP27A* gene transcript when evaluated by Northern analysis (Guo *et al.* 1991; Strugnell *et al.* 1995) although we presently report very low *CYP27A* level (Figure 6) in the Hep3B cell line available in our laboratory. Moreover, recent studies have shown that in the mouse null for the *CYP27A* gene (Rosen *et al.* 1998), normal

concentrations of 25OHD and $1,25(OH)_2D_3$ are observed indicating that, in the mouse, as in other species, D_3-25 hydroxylase(s) other than CYP27A are most likely to be present in the basal state (Gascon-Barré, 1997; Postlind *et al.* 1997) but most particularly following treatment with drugs known to be inducers of cytochromes P450 such as drugs of the anticonvulsant family (Gascon-Barré *et al.* 1986; Mawer, 1979). In 1994 and 1995, two pediatric cases were reported where two sets of siblings were shown to have low circulating 25OHD concentrations despite normal vitamin D intake. In these two case-reports, 25OHD concentrations were shown to increase upon treatment with pharmacological concentrations of vitamin D or 25OHD (Casella *et al.* 1994; Nützenadel *et al.* 1995). Unfortunately, in these two cases, the *CYP27A* genotype was not characterized, the serum cholesterol concentrations were not reported and the subjects were too young to exhibit the clinical manifestations of cerebrotendinous xantomathosis (Rowland, 1995) precluding any meaningful conclusion on the importance of CYP27A or of other as yet unidentified enzyme(s) in the hydroxylation of vitamin D at C-25 while the reported porcine microsomal *CYP25* (Postlind *et al.* 1997; Gill *et al.* 2000) does not seem to be expressed in the human liver.

In conclusion, our data show that *CYP27A* is present not only in the human adult liver but also in the adult kidney as well as in the fetal liver and kidney. They also illustrate that *CYP27A* may be modulated by the vitamin D_3 status as suggested by an increase in the level of its transcript during the summer months. Our observations also indicate that *CYP27A* expression can be significantly upregulated in certain pathological situations such as in hepatic carcinoma and that the neoplastic tissue could contribute to the circulating 25OHD concentration.

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TABLE 1

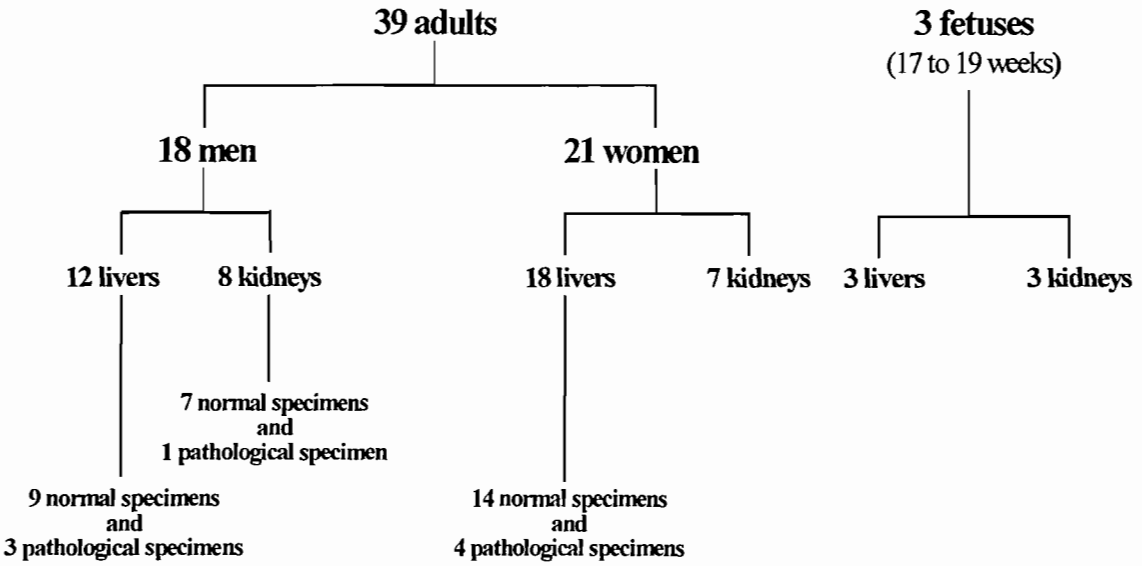
CIRCULATING BIOCHEMICAL VALUES IN ADULT VOLUNTEERS

	WOMEN MEAN ± S.E.M.	MEN MEAN ± S.E.M.	P VALUE
Total calcium (2.23-2.62 mmol/l)	2.2±0.1	2.3±0.1	0.2
Phosphate (0.7-1.3 mmol/l)	1.0±0.1	1.1±0.1	0.44
Bilirubin (2.1-17.1 μmol/l)	15.3±6.2	14.6±1.6	0.93
AST (12-32 U/l)	82.7±42.0	27.7±3.5	0.31
ALT (0-39 U/l)	61.3±26.4	19.1±3.5	0.22
25(OH)D (35-150 nmol/l)	45.5±7.2	40.8±6.2	0.82
$1,25(OH)_2D$ (33-133 pmol/l)	99.0±14.6	119.0±15.6	0.34
Ionized calcium (1.19-1.34 mmol/l)	1.16±0.06	1.16±0.04	0.99
Albumin (34-51 g/l)	33.2±2.4	34.3±2.5	0.75
Alkaline phosphatase (25-97 U/l)	68.9±5.6	122.2±35.6	0.09

Data are presented as means ± S.E.M. Normal reference values are presented in parenthesis. Statistically significant differences between group means were evaluated by the Student's "t" test.

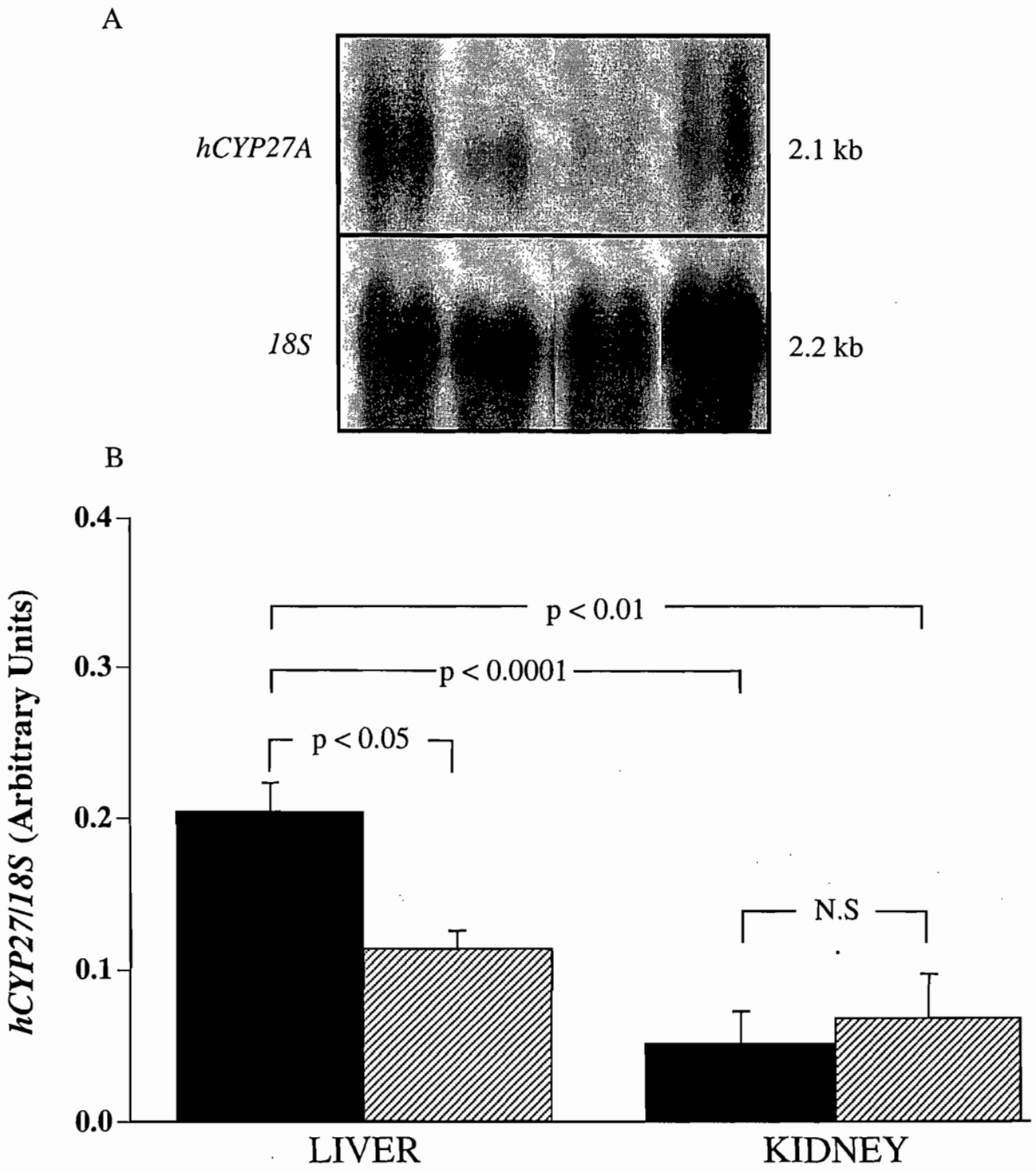
LEGEND TO FIGURE 1

Specimens were obtained from adult and fetal livers and kidneys. In adults, the mean age was: in men 58.1 ± 2.9 years (37 to 73) and in women 57.1 ± 3.7 years (30 to 86). Fetal specimens were obtained between the 17^{th} and 19^{th} week of gestational age. Experimental protocols were approved form the Institutional Human Ethics Review Board and informed consent was obtained from all volunteers.


39 adults
3 fetuses
(17 to 19 weeks)
18 men
21 women
12 livers
8 kidneys
18 livers
7 kidneys
3 livers
3 kidneys
7 normal specimens
and
1 pathological specimen
9 normal specimens
and
3 pathological specimens
14 normal specimens
and
4 pathological specimens

LEGEND TO FIGURE 2

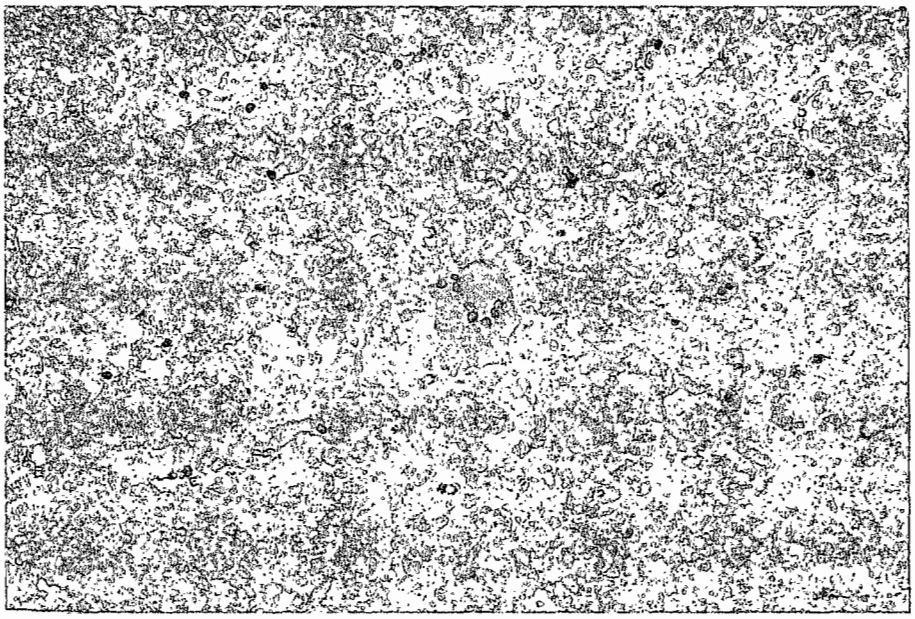
Steady state expression of the gene encoding *hCYP27A* in specimens of normal parenchyma obtained from adult ■ and fetal ▨ livers and kidneys. A. Representative Northern analysis of the *hCYP27A*. B. Means and S.E.M. for the expression of the *hCYP27A* relative to the expression of the *18S* ribosomal gene. Statistically significant differences between group means were evaluated by the Student's "t" test. Liver: adult, n = 27, fetal, n = 3. Kidney: adult, n = 14, fetal, n =3.


A
hCYP27A
2.1 kb
18S
2.2 kb
B
hCYP27/18S (Arbitrary Units)
0.4
0.3
0.2
0.1
0.0
p < 0.01
p < 0.0001
p < 0.05
N.S
LIVER
KIDNEY

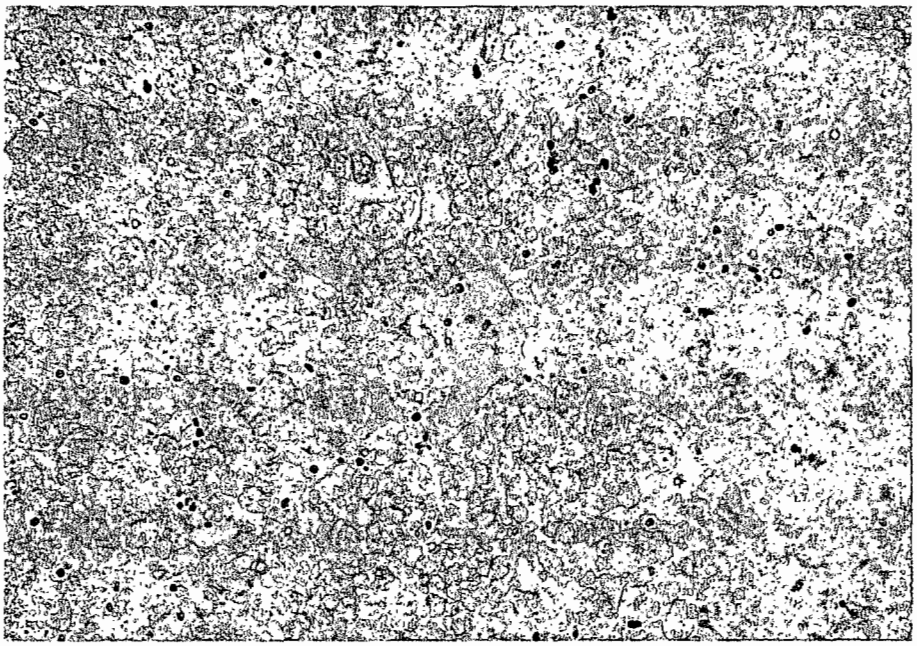
LEGEND TO FIGURE 3

Photomicrographs of a human liver section obtained from a 30 years old woman showing a perivenous area of the liver acinus. A. *In situ* hybridization was carried out using an *hCYP27A* anti-sens riboprobe following *in situ* RT-PCR (10 cycles). Note the accumulation of silver grains around the venous area. B. Adjacent RNase-treated liver section prior to RT-PCR and *in situ* hybridization.

A



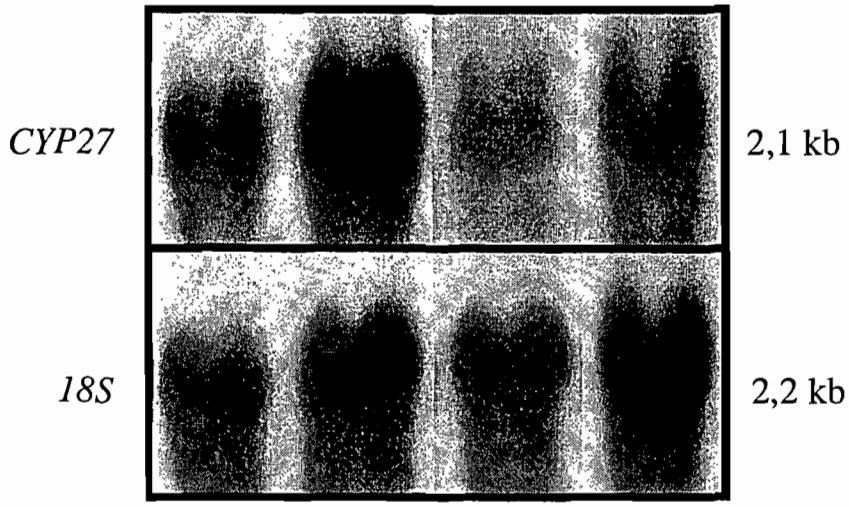
B



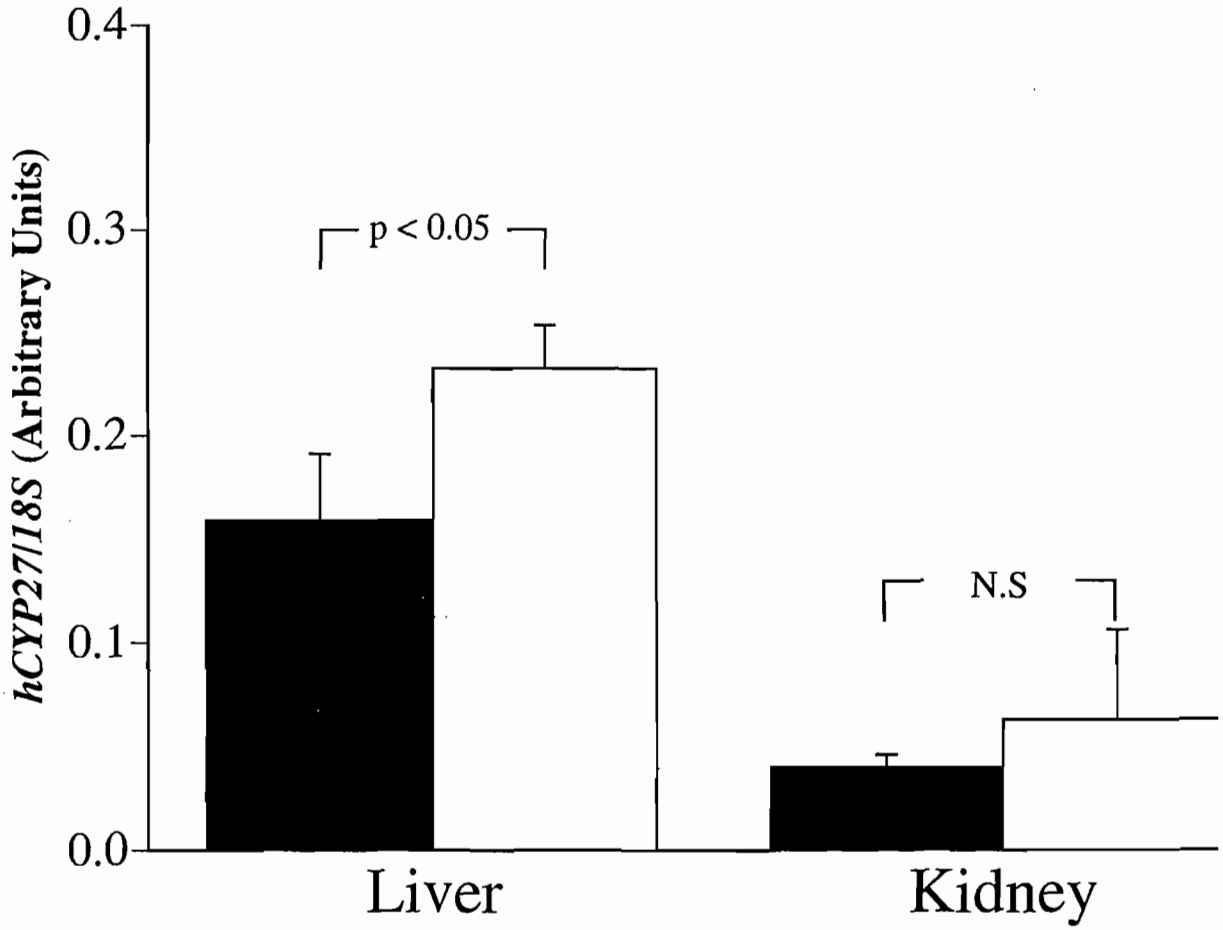
LEGEND TO FIGURE 4

Comparative steady state expression of the gene encoding *hCYP27A* in normal parenchyma of liver and kidney specimens in men ■ and women □. A. Representative Northern analysis of the *hCYP27A*. B. Means and S.E.M. for the expression of the *hCYP27A* relative to the expression of the *18S* ribosomal gene. Statistically significant differences between group means were evaluated by the Student's "t" test. Liver: men, n = 9, women, n = 14; kidney: men, n = 7, women, n = 7.

A

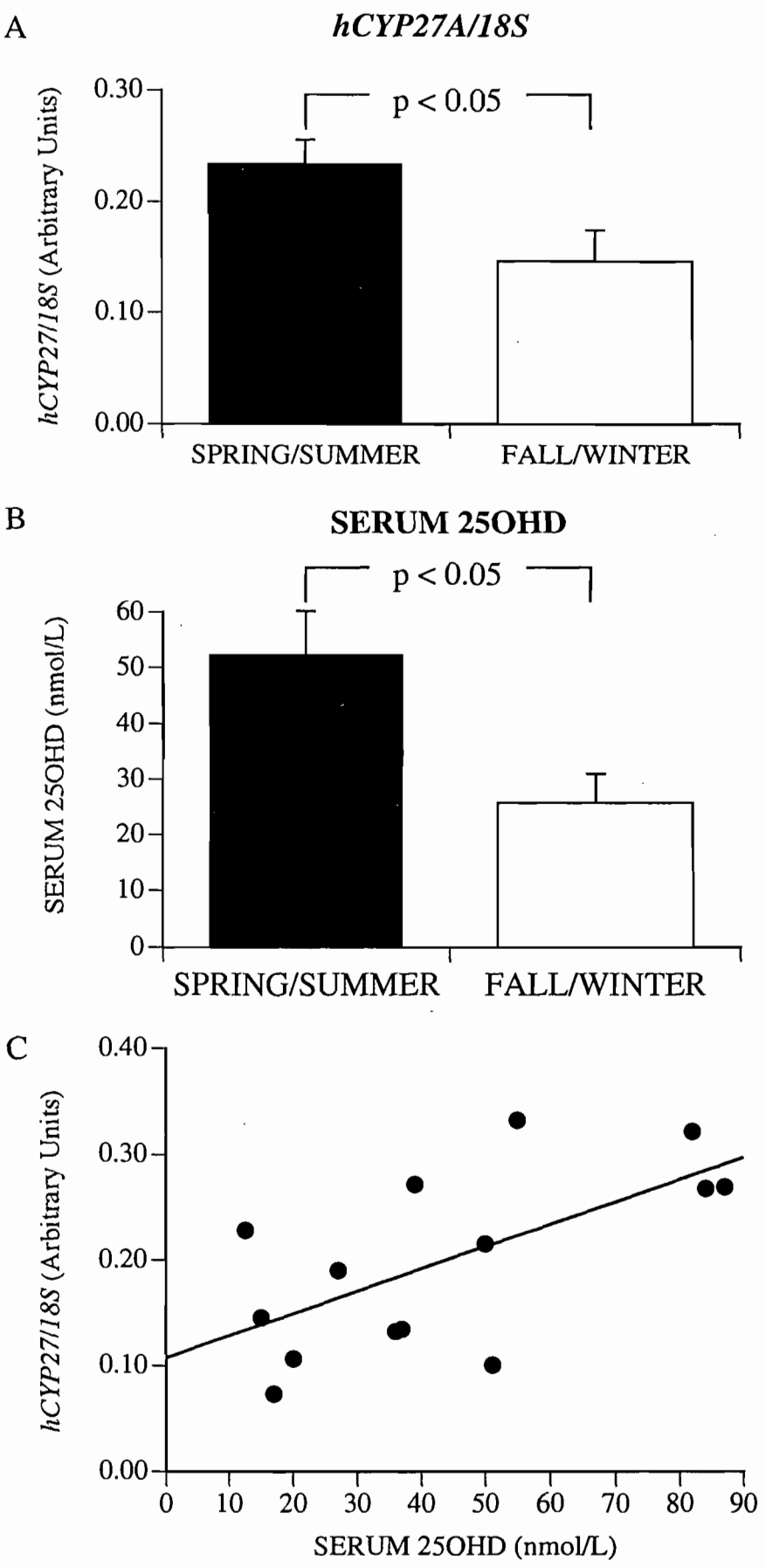

CYP27
2,1 kb
18S
2,2 kb

B


hCYP27/18S (Arbitrary Units)
0.4
0.3
0.2
0.1
0.0
p < 0.05
N.S
Liver
Kidney

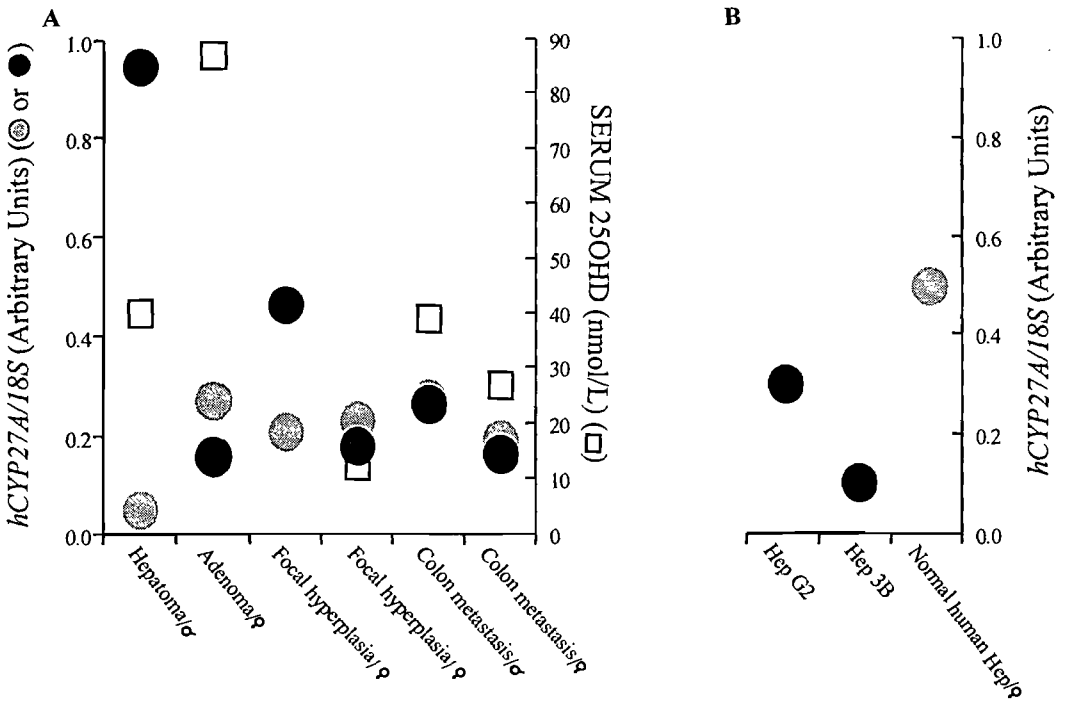
LEGEND TO FIGURE 5

Steady state expression of the gene encoding *hCYP27A* in specimens of normal adult livers and the circulating 25OHD concentrations in spring/summer (April 15th to November 14th) and in fall/winter (November 15th to April 14th). In all cases, *hCYP27A* and circulating 25OHD concentrations were measured in the same subject. A. Mean expression of the *hCYP27A* relative to the expression of the *18S* ribosomal gene. B. Mean serum 25OHD concentrations. C. Pearson correlation coefficient observed between the level of the *hCYP27A* and the circulating 25OHD. $R^2 = 0.41$, $p<0.05$. Statistically significant differences between group means were evaluated by the Student's "t" test. Summer, n = 15, winter, n = 7.


A
hCYP27A/18S
hCYP27/18S (Arbitrary Units)
0.30
0.20
0.10
0.00
p < 0.05
SPRING/SUMMER
FALL/WINTER
B
SERUM 25OHD
SERUM 25OHD (nmol/L)
60
50
40
30
20
10
0
p < 0.05
SPRING/SUMMER
FALL/WINTER
C
hCYP27/18S (Arbitrary Units)
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
90
SERUM 25OHD (nmol/L)

LEGEND TO FIGURE 6

Steady state expression of the gene encoding *hCYP27A* in A. Paired specimens obtained from normal ◎ and pathological ● areas adult livers, and B. in the liver cell lines Hep3B (hepatocellular carcinoma) and HepG2 (hepatoblastoma), and in normal isolated human hepatocytes. *hCYP27A* was evaluated by Northern analysis and values are presented relative to the expression of the *18S* ribosomal gene. □ indicates the serum 25OHD concentrations.


A
hCYP27A/18S (Arbitrary Units) (● or ◎)
SERUM 25OHD (nmol/L) (□)
1.0
0.8
0.6
0.4
0.2
0.0
90
80
70
60
50
40
30
20
10
0
Hepatoma/♂
Adenoma/♀
Focal hyperplasia/♀
Focal hyperplasia/♀
Colon metastasis/♂
Colon metastasis/♀
B
hCYP27A/18S (Arbitrary Units)
1.0
0.8
0.6
0.4
0.2
0.0
Hep G2
Hep 3B
Normal human Hep/♀

2.2.3 Additional Results

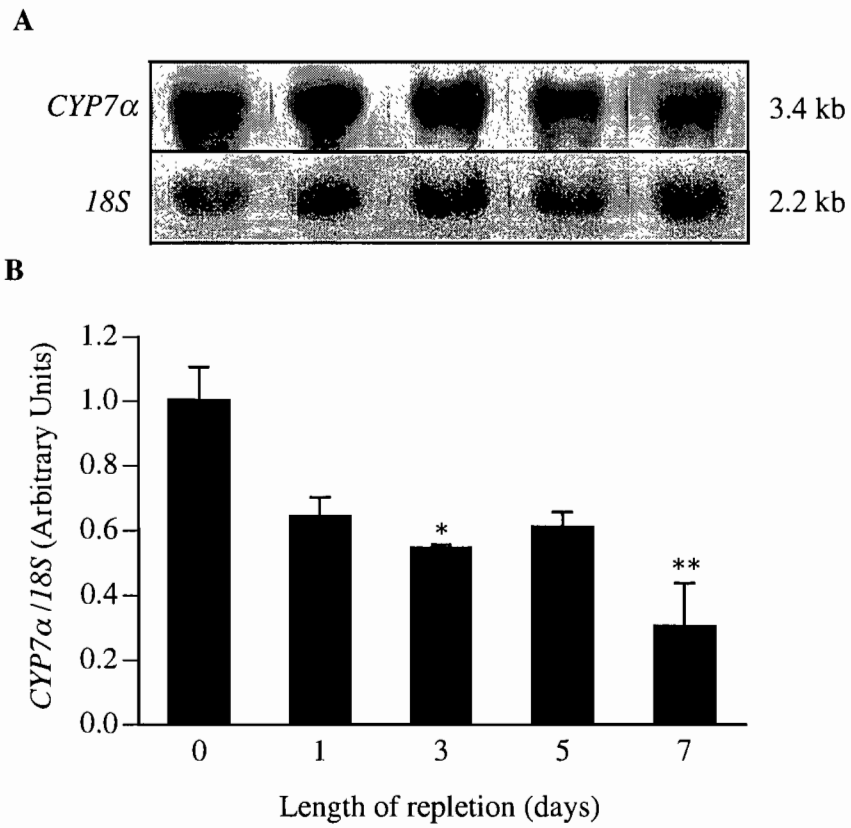


Figure 2.1: Effect of $1,25(OH)_2D_3$ on *CYP7*α (cholesterol 7α-hydroxylase) mRNA steady state levels. Animals were implanted with osmotic mini-pumps releasing 28pmol/day of $1,25(OH)_2D_3$, and sacrificed following 1, 3, 5 and 7 days of treatment. RNA was extracted from liver and mRNA levels were assessed as described in Article 1. A cDNA CYP7α probe was generated specifically for this study by RT-PCR using the primers described in the study by Twisk *et al.* (227). A. Representative Northern analyses of the CYP7α gene transcript. B. Densitometric analyses. Data are presented as means ± S. E. M. Statistically significant differences between group means were analysed by ANOVA with individual contrasts evaluated by the Bonferonni test. Overall ANOVA $p<0.02$. Significantly different from D-Ca-, *$p<0.03$, **$p<0.0001$; n=3 animals/group.

Results: *CYP7α* mRNA levels were decreased by 28% (NS) starting on the 1st day of $1,25(OH)_2D_3$ treatment. Levels further decreased by 38% ($p<0.03$) by day 3 and by 68% at the end of the 7 day treatment ($p<0.001$).

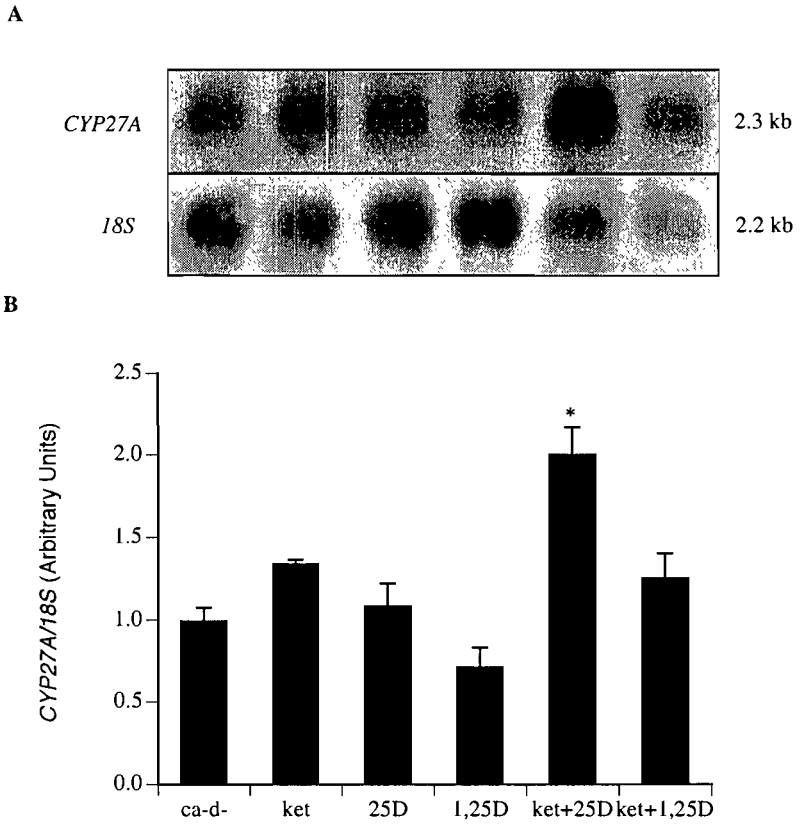


Figure 2.2: Effect of ketoconazole alone or in combination with vitamin D metabolites on *CYP27A* steady state levels.

Animals were injected with 1.75mg/kg i.p. of ketoconazole daily, 2 days before beginning supplementation for 3 days with 28pmol/d 25(OH)D_3 or 1,25(OH)D_3 by osmotic mini-pump at which time ketoconazole injections continued to be administered. A. Representative Northern analyses of the *CYP27A* gene transcript. B. Densitometric analyses. Data are presented as mean ± S. E. M. Statistically significant differences between group means were analysed by ANOVA with individual contrasts evaluated by the Bonferonni test. Main effect $p<0.0005$, * $p<0.0002$, n=3 animals/group.

Results: Ketoconazole administration alone, or administered together with $1,25(OH)_2D_3$ produced a slight stimulatory effect on *CYP27A* steady state levels compared to D-Ca- rats (10% and 25% respectively, NS). $25(OH)D_3$ treatment increased steady state levels by 8% (NS) whereas $1,25(OH)_2D_3$ decreased levels by 29% (NS). Ketoconazole in conjunction with $25(OH)D_3$ increased *CYP27A* mRNA levels 2 fold ($p<0.0002$). Circulating $25(OH)D_3$ levels were in the order of less than 10 nmol/L as reported in Article 3 among all groups. Administration of ketoconazole did not produce significant changes in $1,25(OH)_2D_3$ concentrations, although a noteworthy elevation from 624±41 pmol/L to 1102±125 pmol/L in the group receiving $1,25(OH)_2D_3$ and ketoconazole was observed compared to that with $1,25(OH)_2D_3$ alone .

2.3 CONCLUSIONS

The data obtained in these studies indicate that hepatic *CYP27A* is affected by the D_3 status in both human and rat. The human gene levels were significantly correlated with circulating levels of 25(OH)D and were higher in the spring/summer compared to the fall/winter. Fetal liver also expressed the *CYP27A* gene transcript, albeit at a much lower level than that observed in the adult. In addition, expression levels were higher in females compared to males, and gene levels were also detectable in pathological states, where levels could be significantly upregulated.

Rat *CYP27A* was affected by a chronic treatment of $1,25(OH)_2D_3$, which proved to have a sharp abolishing effect on the transcription rate, whereas the half-life was unaffected. D_3 and $25(OH)D_3$ were without any effect. In parallel, the rat *CYP7α* mRNA levels were also sensitive to only $1,25(OH)_2D_3$ repletion. Hepatocytes showed the highest level of expression in *CYP27A* mRNA levels, although the gene was also detected in stellate, Kupffer and endothelial cells. As with the human *CYP27A*, rat *CYP27A* was significantly higher in female compared to male rats. The signal localization was found in both the PV and PP areas in the rat, whereas the human *CYP27A* gene transcript was mainly localized only in the PV region of the liver acinus.

CHAPTER 3: EXPERIMENTAL SECTION (STUDIES IN INTESTINE)

3.1 EFFECT OF THE VITAMIN D_3 HORMONAL AND NUTRITIONAL STATUS ON CYP27A, THE INTESTINAL VITAMIN D_3 25-HYDROXYLASE

3.1.1 Preliminary findings

In the intestine, $1,25(OH)_2D_3$ elicits both genomic, as exemplified by the synthesis of calbindins (229) and non-genomic responses, involving rapid increases in intracellular calcium concentration, cGMP levels and altered phospholipid synthesis (230;231). Although the role of D_3 in the intestine has been directly and mainly linked to the absorption of calcium and phosphorus, it has also been shown that D_3 plays a role in intestinal membrane integrity and physiology (232). In addition, in the human fetal jejunum, $1,25(OH)_2D_3$ acts as a hormonal agent by controlling cellular proliferation and differentiation (233).

Of the hydroxylases involved in the activation and metabolic pathways of D_3, all three major hydroxylation activities (C-25, C-24 and C-1α) have been reported and described in the intestine.

The presence of CYP27A, a cytochrome principally involved in the metabolism of biliary acids, but also capable of hydroxylating D_3 at C-25, has been reported in mouse intestine (234).

1α-hydroxylase activity has been described in CaCo-2 cells (235) which is the cell line best resembling morphologically the small intestinal absorptive cells (236;237). Recently, the gene has also been identified in fetal and adult rat intestine (238).

The 24-hydroxylase system has been extensively studied in intestine, and shown to display an induction profile responsive to D_3 supplementation *in vivo* (96;239).

3.1.2 Hypotheses

The presence of the three hydroxylases in the intestine, suggests local $25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ production and evokes the possibility of the intestine being an organ responding to D_3 and/or its metabolites via an autocrine/paracrine mode of action. Moreover, the levels of $1,25(OH)_2D_3$ are tightly regulated in the body, depending on calcium and phosphorus status. This regulation has been proposed to be at the level of the D_3 hydroxylases (240), and is thought to be mediated by the VDR.

3.1.3 Objectives

- Investigate the effect of the D_3 hormonal and nutritional status on the rat intestinal *CYP27A* in our D-Ca- rat model.
- Evaluate the presence of *CYP27A*, *CYP27B1*, *CYP24* and *VDR* along the human fetal intestine and colon and investigate the effect of $1,25(OH)_2D_3$ on their expression levels.

3.2 RESULTS

3.2.1 Article 3: 1,25-Dihydroxyvitamin D_3 downregulates the rat intestinal vitamin D_3 25-hydroxylase CYP27A.

Article published in Am J Physiol Endocrinol Metab. 2001 Aug;281(2):E315-25.

(ARTICLE 3)

1,25-DIHYDROXYVITAMIN D_3 DOWN-REGULATES THE RAT INTESTINAL VITAMIN D_3-25 HYDROXYLASE *CYP27A*

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Short title: Intestinal D_3-25 hydroxylase

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ABSTRACT

The vitamin D_3-25 hydroxylase CYP27A is predominantly located in liver but its expression is also detected in extrahepatic tissues. Our aim was to evaluate the regulation of *CYP27A* by vitamin D_3 (D_3) or its metabolites in rat duodena. D depleted rats were repleted with D_3, $25OHD_3$, or $1,25(OH)_2D_3$, or acutely injected $1,25(OH)_2D_3$ to investigate the mechanisms of action of the hormone. All D_3 compounds led to a progressive decrease in *CYP27A* mRNA with levels after D_3 representing 20% of that observed in D depletion. $25OHD_3$ decreased *CYP27A* mRNA by 55% while $1,25(OH)_2D_3$ led to a 40% decrease which was accompanied by a 31% decrease in CYP27A protein levels and an 89% decrease in enzyme activity. Peak circulating $1,25(OH)_2D_3$ concentrations were, however, the highest in D_3-, followed by $25OHD_3$- and $1,25(OH)_2D_3$-repleted animals. $1,25(OH)_2D_3$ resulted in both a decrease in *CYP27A* mRNA half-life and transcription rate. Our data illustrate that the intestine expresses the D_3-25 hydroxylase and that the gene is highly regulated *in vivo* through a direct action of $1,25(OH)_2D_3$ or through the local production of D_3 metabolites.

Key words: Intestinal D_3-25 hydroxylase, *CYP27A*, vitamin D_3, $1,25(OH)_2D_3$, $25OHD_3$.

INTRODUCTION

Vitamin D_3 (D_3) of endogenous origin or ingested vitamin D_2 (D_2) (collectively referred to as vitamin D (D)) are natural secosteroids that have, in their native forms, no biological activity. D exhibits a short circulating half-life and is efficiently captured by storage sites such as adipose tissues and muscles, and by the liver where the vitamin undergoes its first anabolic biotransformation through a C-25 hydroxylation reaction. The hepatic product 25-hydroxyvitamin D (25OHD) is rapidly exported to the systemic circulation where its half-life has been reported to be in the order of 2-3 weeks in humans. This long half-life associated with the efficient hepatic capture and hydroxylation of the parent compound makes 25OHD the most reliable marker of the vitamin D nutritional status in humans and laboratory animals . At physiological concentrations, 25(OH)D is not, however, known to have any significant biological activity but must undergo regulated-hydroxylation steps at C-1α or C-24 in the kidney to achieve full biological action through either its hormonal form 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D), or the now active bone and cartilage candidate 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2$D) (39). Other vitamin D compounds such as 1α-hydroxyvitamin D_3 (1$\alpha$$OHD_3$) and dihydrotachysterol must also undergo the necessary C-25 hydroxylation step to acquire their full biological activity (11,26,41).

To date, two independent mono-oxygenase systems active on the C-25 hydroxylation of D_3 or D_2 family of compounds have been described. DeLuca's group (9) first reported the enzyme to be located in the hepatic microsomal fraction while a few years later, Bjorkhem and Holmberg (10) reported that the mitochondrial sterol-27 hydroxylase exhibited enzyme activities towards the C-25 hydroxylation of D_3. The molecular identity of the human or rat microsomal D-25 hydroxylase has not yet been reported while the mitochondrial enzyme has been cloned in several species and the gene encoding the enzyme termed *CYP27A* (3,14,26,49,54).

Studies on the kinetics of the hepatic D_3-25 hydroxylases have demonstrated that the affinity of the microsomal enzyme is much higher (as indicated by a significantly lower Km) than that of the mitochondrial enzyme, an observation which has led to the conclusion that the microsomal D_3-25 hydroxylase is most likely physiologically more relevant than its mitochondrial counterpart (22). The intestine, however, is likely to be exposed to

significantly higher concentrations of D compounds than the liver. Indeed, both the vitamin of dietary or pharmacological origin will be presented to the small intestine while only a fraction of the vitamin of either endogenous or exogenous origin will be captured by the liver (24,25). These observations strongly indicate that, in the small intestine, the physiological significance of the mitochondrial D-25 hydroxylase may be highly relevant. Interestingly, the small intestine has been reported to express *CYP27A* (3), and to also exhibit enzyme activities related to the hydroxylation of D compounds at C-24 (5,36,47) while the Caco-2 cell line (a line closely resembling the small intestine) (30) has been reported to be able to hydroxylate $25OHD_3$ at C-1α (16) indicating that the small intestine, in addition to its response to the D_3 endocrine system, may be fully able to regulate its own D metabolism and to respond to its local D-dependent needs through an auto- and/or intracrine process.

To date, studies on the regulation of the *CYP27A* gene products have focussed solely on its significance and importance in relation to the biosynthesis of bile acids, and the molecular mechanisms by which *CYP27A* is regulated by D_3 or its metabolites, most particularly in intestine, are presently unknown. The aims of the studies were, therefore, to determine the effect of an *in vivo* exposure to vitamin D_3, $25(OH)D_3$, or $1,25(OH)_2D_3$ on the level of the *CYP27A* gene transcript in rat duodena, and to evaluate the mechanisms by which $1,25(OH)_2D_3$ regulates its expression.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The influence of the vitamin D endocrine system on the steady-state expression of the duodenal *CYP27A* was studied in D depleted rats repleted with either calcium alone, D_3, $25OHD_3$ or $1,25(OH)_2D_3$. In order to investigate the mechanism of action by which $1,25(OH)_2D_3$ influenced the expression of *CYP27A*, studies were conducted on the half-life as well as the transcription rate of the gene. The effect of known cytochrome P-450 inducers on the steady-state levels of *CYP27A* mRNA was also evaluated.

ANIMALS

All animals used during the experiments were treated according to the standards of ethics for animal experimentation of the Canadian Council on Animal Care. All protocols were approved by the local animal ethics committee.

Studies on the characterization of the *CYP27A* fragment generated in our laboratory were done in normal male Sprague-Dawley rats (50). Hypocalcemic-vitamin D depleted male rats (D-Ca-) were obtained as previously described (21,27). Animals were then submitted to experimental protocols aimed at achieving an *in vivo* repletion with dietary calcium alone or with physiological concentrations of D_3, $25OHD_3$ or $1,25(OH)_2D_3$ as described below.

REPLETION WITH CALCIUM, D_3, $25(OH)D_3$, OR $1,25(OH)_2D_3$

Expression of the *CYP27A* duodenal gene transcript was first studied under steady-state conditions in D depleted animals and in animals repleted with calcium alone, or with D_3 compounds. Repletion with calcium alone was achieved by an oral supplementation with a 3% calcium gluconate solution as drinking water for a period of 7 days as previously described (21,27). Repletion with D_3 compounds was achieved by the intraperitoneal implantation of mini-osmotic pumps (Alza Corporation, Palo Alto, CA, USA) containing either D_3 at a dose of 6.5nmol/day, $25(OH)D_3$ at a dose of 28 pmol/day, or $1,25(OH)_2D_3$ also at a dose of 28 pmol/day (21,27). All compounds were administered in vehicle containing 95% ethanol:propylene glycol:0.9% saline, 3:13:4, v/v/v. At the time of mini-pump

implantation a loading dose of 3.2 nmol D_3, 14 pmol $25(OH)D_3$, or 14 pmol $1,25(OH)_2D_3$ was administered i.p. in order to rapidly raise serum concentrations of D_3, $25(OH)D_3$, or $1,25(OH)_2D_3$ and hence accelerate the establishment of steady state conditions. D depleted animals were implanted with mini-osmotic pumps containing vehicle only. Repleted rats were given a 0.5% calcium gluconate solution as drinking water while D-depleted controls received demineralized water. Animals repleted with D_3 were killed after one week of repletion while animals repleted with $25OHD_3$ or $1,25(OH)_2D_3$ were killed 1, 3, 5 or 7 days following initiation of the repletion protocol. At the time of euthanasia the animals were between 7-8 weeks of age.

TREATMENT WITH CYTOCHROME P-450 INDUCERS

Studies on the induction of the gene encoding *CYP27A* were achieved using xenobiotics known to induce cytochrome P-450 izozymes. Normal male rats were exposed to dexamethasone (one single i.p. injection: 400mg/kg), 3-methylcholanthrene (one single i.p. injection: 30 mg/kg), β-naphtoflavone (three daily i.p. injections: 80mg/kg), acetone (1% v:v in drinking water for a period of 10 days), or phenobarbital (350 mg/ml in drinking water for a period of 10 days) (40).

HALF-LIFE OF THE *CYP27A* GENE TRANSCRIPT

Studies on the half-life of the *CYP27A* gene transcript were achieved in D depleted rats subjected to a single i.v. dose (0, 2.4, 12, 120, or 240 nmol/kg) $1,25(OH)_2D_3$. Pharmacological hormonal concentrations were used in order to rapidly achieve the $1,25(OH)_2D_3$ effect on the *CYP27A* gene before the production of significant down stream metabolites of the hormone. A single i.p. dose of 0.5mg/kg actinomycin D dissolved in 95% ethanol:saline (1:1 v:v) was administered 3 hours after exposure to $1,25(OH)_2D_3$ and animals were sacrificed prior to and 1, 3, or 6 hours after actinomycin D administration. *CYP27A* mRNA levels were evaluated as described below.

TRANSCRIPTION RATE OF THE GENE ENCODING *CYP27A*

The transcription rate of the gene encoding *CYP27A* was evaluated in duodenal nuclei obtained from either hypocalcemic D_3 depleted rats or from rats exposed to a single i.v dose

of 120 nmol/kg $1,25(OH)_2D_3$ 6 hours before euthanasia. Nuclear run-on assays were done as indicated below.

25-HYDROXYLASE ACTIVITY

The mitochondrial 25-hydroxylase activity was measured in freshly isolated duodenal mitochondria obtained from D-depleted rats and from animals repleted by intraperitoneal mini-osmotic pumps containing 28 pmol/day $1,25(OH)_2D_3$ for a period of 7 days as described above. 1α-hydroxyvitamin D_3 (1αOHD_3) (Leo Pharma Inc., Ajax, Ontario, Canada) was used as substrate.

Duodenal mucosal cells were gently scrapped off and mitochondria were isolated as described by Rosenberg and Kappas (42). The final mitochondrial pellet was resuspended in 0.25 M sucrose, 10 mM Tris, 10 mM KCl, 1 mM EDTA, heparin 3 U/ml, pH 7.4. Protein concentration was determined according to Bradford (13). The incubation reaction (0.4-0.6 mg protein in 1.0 mL) contained 40 mM potassium phosphate, 0.25 M sucrose, 200 μM EDTA, 20 mM $MgCl_2$, 0.2 mg BSA, 2 μg N,N'diphenylphenylelenediamine (Aldrich Chem. Co., Milwaukee, WI), 10 mM isocitric acid (Sigma Chemical Co., St. Louis, MO), pH 7.4. The reaction was started with 20 nmol 1αOHD_3 and allowed to continue for 40 min at 37°C under gentle shaking. Blank reactions were carried out with boiled mitochondria. The reaction was terminated with 3.75 mL chloroform:methanol (1:2 v:v), and 6000 cpm [^{3}H]$1,25(OH)_2D_3$ was added to correct for recovery during the extraction and chromatographic procedures. Reaction mixtures were extracted twice as described by Bligh and Dyer (12). After extraction and evaporation, the residue was dissolved in 150 μL hexane and injected into a Beckman model 160 HPLC (Beckman Instruments, Palo Alto, CA) fitted with a Zorbax-Sil column (4.6 x 250 mm) (Dupont Instruments, Wilmington, DE) and eluted in hexane:isopropanol (9:1 v:v) at a flow rate of 2mL/min. Metabolites were detected at 254 nm. The fractions corresponded to crystalline $1,25(OH)_2D_3$ (retention time 15 min. without overlap from 1αOHD_3) were collected and counted (Beta LS1801 spectrometer, Beckman Instrument, Palo Alto, CA). The identity of the product formed was confirmed by a second HPLC on a C-18 column eluted with hexane:isopropanol (8:2 v:v).

EXPERIMENTAL PROCEDURES

Determination of Circulating Ionized Calcium and Vitamin D Metabolites

Serum Ca^{2+} concentrations were measured with an ICA2 ionized calcium analyser (Radiometer, Copenhagen, Denmark). Serum $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations were measured using the Incstar 25(OH)D and $1,25(OH)_2D$ RIA assay kits (Incstar Corporation, Stillwater, Minnesota, USA) according to the manufacturer's instructions.

Molecular Biology Procedures

At the time of euthanasia, the duodena and livers were isolated and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Total intestinal RNA was extracted, blotted onto nylon membranes (Qiagen, Mississauga, Ontario, Canada) and processed for Northern analyses as previously described (35). The radiolabeled *CYP27A* probe was a cDNA fragment generated specifically for the present studies by RT-PCR. The 404 bases corresponded to base pairs 399 to 803 within the N-terminal coding region of the rat gene based on the published sequence of Su *et al.* (49), (Genbank accession #M38566). The primers 5'TCTCTGGCTCTAAACTCTTGGC3' and 5' CTCGTGAAGTGCAGCACATA 3' used were custom synthesised by the Sheldon Biotechonology Centre (McGill University, Montreal, Quebec, Canada). The following PCR program was used for 30 cycles: 30s at 65°C, 10s at 72°C and 30s at 95°C. The fragment obtained was cloned into the vector PCRII (InVitrogen, Carlbad, CA, USA) and sequenced in order to confirm identity. The functional specificity of the *CYP27A* fragment generated revealed higher mRNA expression in female than in male liver and a significant hepatic induction of the gene transcript by dexamethasone (50) as expected from previous studies (1,2,4,48).

The *CYP24* probe was a 247 base rat renal cDNA insert from the KpnI site of the pUC19 vector (38), and the *18S* ribosomal RNA was a 1.5 kb human cDNA insert from the EcoRI site of pBluescript SK^- vector (ATCC #77242). Probe labelling, blot hybridization, washing, exposure and photodensitometric evaluation were performed as previously described (18,35).

Nuclear run-on transcriptional assay

Nuclei were isolated from duodena of D-Ca- or of $1,25(OH)_2D_3$ repleted rats by the

method of Widnell and Tata (56) using successive sucrose gradient centrifugations. The nuclei obtained were resuspended in storage buffer (40% glycerol, 5 mM $MgCl_2$, 10 mM Tris pH 7.4, 1 mM DTT and 1 mM EDTA) and stored at -80°C. The rate of *CYP27A* gene transcription was measured using a previously described nuclear run-on transcriptional assay (43) with the following modifications. Nuclei were pelleted by centrifugation and resuspended in 50μL of nuclear run-off reaction mixture (50 mM Tris pH 7.5, 50 mM $MgCl_2$, 2 mM DTT, 2 mM spermidine, 25U Rnase inhibitor, 1 mM ATP, 1 mM CTP, 1 mM GTP, and 50 μCi [^{32}P] UTP (3000Ci/mmol, Amersham Pharmacia Biotech, Baie D'Urfé, Quebec, Canada) and incubated at 30°C for 60 min. The labelled RNA was hybridized to nylon membranes on which 300 ng of the 404 bp D_3-25-hydroxylase cDNA fragment with 150 ng of 18S ribosomal RNA cDNA fragment (positive control), and 100 ng of pBS (negative control) had been dotted and hybridized in 5% SDS, 400 mM $NaPO_4$ pH 7.2, 1 mM EDTA, 1 mg/ml BSA, 50% formamide and 240 μg/ml of salmon sperm DNA. The membranes were prehybridized for 4 hours at 52°C in hybridization solution without labelled RNA then hybridization was performed at 52°C for 72 hours. The membranes were washed and exposed to x-ray films for 14 days and densitometry was performed as previously described (18,35).

Western analyses of the CYP27A protein

The relative levels of CYP27A protein was determined by Western blot analyses. Membranes from intestinal samples were disrupted by sonication and homogenized in 100 mM TRIS pH 7.6, 3mM PMSF, 300 mM KCl and 1% BSA, centrifuged at 100 000g, and the supernatant was precipitated with 40% ammonium sulfate overnight. 30 μg of proteins were loaded onto a SDS-PAGE 5-15% gradient acrylamide gel and transblotted on PVDF membranes. The membranes were first incubated for 1h with a rabbit polyclonal antibody raised against human CYP27A 1:1000 (gift from Dr. David Russel, University of Texas Southwestern Medical Center, Dallas,TX), followed by an incubation with an anti-rabbit IgG streptavidin-biotinylated species specific antibody 1:1000 (Amersham Pharmacia Biotech, Baie D'Urfé, Quebec, Canada), and finally incubated with a streptavidin-biotinylated horseradish-peroxidase complex 1:1000 (Amersham Pharmacia Biotech, Baie D'Urfé, Quebec, Canada). The antigen-antibody complex was visualized with 3,3'-diaminobenzidine (Sigma Chemical Co., Mississauga, Ontario, Canada). Quantification was achieved by

densitometric scanning.

STATISTICAL ANALYSES

Data are presented as means ± SEM. Statistically significant differences between group means were evaluated by ANOVA, or the Student's "t" test as indicated in the figure legends. Individual between-group contrasts were evaluated using the Bonferroni test.

RESULTS

RELATIVE INTESTINAL *CYP27A* LEVEL AND DRUG INDUCIBILITY

Figure 1A and B presents the relative level of *CYP27A* expression in normal male rat liver and duodenum. As illustrated, the steady-state level of *CYP27A* mRNA was found to be 3 fold higher in liver than in duodenum ($p<0.0001$). A survey of other parts of the intestine indicates values relative to those found in duodenum of 85%, 69% and 77% in jejunum, ileum and colon respectively.

In order to investigate the modulation of the duodenal *CYP27A* transcript by known pharmacological agents, animals were treated with acetone, phenobarbital, β-naphtoflavone, 3-methylcholanthrene, and dexamethasone. As illustrated in Figure 2A and B, only dexamethasone was found to significantly increase (5 fold induction) *CYP27A* mRNA expression in intestine ($p<0.0001$). Finally, the effect of the calcium and vitamin D status on the relative abundance of the *CYP27A* gene transcript was investigated. As illustrated in Figure 2C and D, *CYP27A* was found to be 2 fold higher in vitamin D depleted than in normal rat duodenum ($p<0.02$) and this irrespective of the calcium status of the animals. Indeed, no significant difference was observed in *CYP27A* gene expression in duodena obtained from hypocalcemic (D-Ca-) compared to that obtained from normocalcemic (D-Ca+) D depleted rats.

EFFECT OF D_3, 25(OH)D_3, OR 1,25$(OH)_2D_3$ REPLETION ON *CYP27A* mRNA LEVEL

The influence of the D status on intestinal *CYP27A* mRNA abundance, prompted investigation on the *CYP27A* gene response to a one week repletion with either the parent compound D_3, or with its hepatic (25OHD_3), or kidney (1,25$(OH)_2D_3$) metabolites.

Repletion with D_3

D_3 was found to significantly influence *CYP27A* mRNA levels with, as illustrated in Figure 3, an 80% decrease in the abundance of the *CYP27A* gene transcript observed after one week of repletion ($p<0.0001$). The dose of D_3 used achieved normalization of the circulating ionized calcium, 25OHD_3 and 1,25$(OH)_2D_3$ after one week of repletion as previously reported (17,21,27). The 1,25$(OH)_2D_3$ concentrations achieved were found to be

1760 ±152 pmol/L at day one of the repletion period and to decrease to 1115 ±128 pmol/L at the time of euthanasia.

Repletion with 25(OH)D_3

The serum ionized calcium concentrations increased steadily from a mean value of 0.82 ± 0.01 mmol/L in D-Ca- to 1.13 ± 0.04 after one week 25OHD_3 administration. Serum 25(OH)D_3 concentrations remained, however, low during the period studied with a mean concentration of 6.5 nmol/L after one week of repletion, whereas serum1,25$(OH)_2D_3$ concentrations increased from an average of 95 ± 15 pmol/L in D depleted animals to an average of 1143 ±110 pmol/L after one week of 25OHD_3 repletion.

As illustrated in Figure 4, the intestinal *CYP27A* gene transcript exhibited a progressive decrease in its level of expression with a non-significant 10% decrease at day one but with a significant 33% decrease after 72 hours of 25(OH)D_3 administration ($p<0.03$). After one week of 25OHD_3 repletion, the mean level of the *CYP27A* transcript was found to be decreased by 55% when compared to the level observed in animals not exposed to 25OHD_3 ($p<0.008$). Moreover, the decrease in *CYP27A* mRNA abundance was shown to be linear over the one week-period studied ($r^2 = 0.787$, $p<0.001$).

Repletion with 1,25$(OH)_2D_3$

The serum ionized calcium concentrations increased steadily from a mean value of 0.73 ± 0.03 to 1.21 ± 0.09 mmol/L throughout the one week of 1,25$(OH)_2D_3$ repletion. Serum 25(OH)D_3 remained unchanged during the course of 1,25$(OH)_2D_3$ administration while 1,25$(OH)_2D_3$ concentrations which averaged 95 ± 15 pmol/L at day zero remained at a plateau during the course of 1,25$(OH)_2D_3$ repletion averaging 603 ± 90 pmol/L after one day of repletion and 503 ± 123 pmol/L after one week of 1,25$(OH)_2D_3$ administration.

As illustrated in Figure 5A and B, 1,25$(OH)_2D_3$ administration resulted in a highly significant 30% decrease in *CYP27A* mRNA levels as soon as 24 hours after the beginning of 1,25$(OH)_2D_3$ administration ($p<0.0008$). *CYP27A* mRNA levels remained thereafter unchanged with an observed 40% decrease ($p< 0.0008$) in *CYP27A* mRNA abundance after one week of 1,25$(OH)_2D_3$ exposure compared to the level observed in rat duodena not exposed to the hormone. Moreover, Western analyses (Figure 5 C and D) revealed a concomitant 31% decrease in CYP27A protein level after one week of 1,25$(OH)_2D_3$

administration ($p<0.003$).

25-hydroxylase activity

Figure 6 illustrates the effect of one-week exposure to 28 pmol/day $1,25(OH)_2D_3$ on the 25-hydroxylase activity is isolated duodenal mitochondria. $1,25(OH)_2D_3$ repletion had a significant influence on CYP27A activity with an average 89% decrease in $1\alpha,25(OH)_2D_3$ production following incubation with $1\alpha OHD_3$ ($p<0.01$).

MECHANISMS OF $1,25(OH)_2D_3$ ACTION

The observation that the decrease in *CYP27A* mRNA were highly and rapidly sensitive to $1,25(OH)_2D_3$ prompted studies on the mechanisms by which the hormone influences the abundance of the *CYP27A* gene transcript. These studies were conducted using an acute model of $1,25(OH)_2D_3$ exposure.

Effect of acute 1,25 $(OH)_2D_3$ administration on the level of the CYP27A gene transcript

Serum ionized calcium concentrations increased only slightly from 0.78 ± 0.01 to a range varying from 0.90 ± 0.01 (2.4 nmol/kg dose) to 1.01 ± 0.03 (240 nmol/kg dose) 6 hour after the i.v. injection of $1,25(OH)_2D_3$. The $1,25(OH)_2D_3$ concentrations reached, at the time of euthanasia, were $9\,277 \pm 551$ pmol/L in rats injected with the 2.4 nmol/kg dose to over 30 000 pmol/L in animals injected with the 12, 120 and 240 nmol/kg doses of $1,25(OH)_2D_3$ ($p<0.001$).

As is illustrated in Figures 7A and B, as soon as 6h after $1,25(OH)_2D_3$ exposure, duodenal *CYP27A* mRNA levels were found to progressively decrease with increasing doses of the hormone (2.4 to 240nmol/kg) whereas *CYP24* mRNA levels, which were used as controls for the $1,25(OH)_2D_3$ response, were found to be concomitantly and highly significantly upregulated (Figures 7C and D).

CYP27A mRNA half-life

As is illustrated in Figure 8, treatment of Ca-D- rats with 0.5mg/kg actinomycin D did not significantly influence *CYP27A* mRNA levels in untreated rats over the 6 hour period studied although a slight, but not significant, increase over basal values was observed at the 6 hour-time period. A dose of 5mg/kg actinomycin D which was also used in order to verify

whether the dose of actinomycin D used in the original experiment was sufficient to halt *CYP27A* gene transcription revealed that *CYP27A* mRNA levels were not influenced for up to six hours following actinomycin D injection when compared to untreated D-Ca- controls. All further studies were, therefore, done using the 0.5 mg/kg dose of actinomycin D. The effectiveness of the dose of actinomycin D used in inhibiting the process of transcription was also verified by examining its effect on the mRNA levels of the gene encoding *CYP24*. A 0.5mg/kg dose given one hour before 1,25 $(OH)_2D_3$ injection was found to effectively prevent the upregulation of the *CYP24* gene transcript (results not shown). Following the i.v. injection of $1,25(OH)_2D_3$, a 24% decrease in the level of the *CYP27A* gene transcript was observed one hour after actinomycin D administration when compared to the level observed in D-Ca- animals. Moreover, the levels of the transcript steadily decreased to 36% of the level observed in animals not subjected to actinomycin D administration, 6 hours after actinomycin D administration ($p<0.03$). When compared to their actinomycin D-paired D depleted controls, the relative abundance of the *CYP27A* gene transcript was found to be decreased by 77% at the 6 hour-time point ($p<0.002$).

CYP27A gene transcription rate

Nuclear transcription run-on assays were performed on nuclei isolated from duodena of hypocalcemic D depleted rats as well as on nuclei obtained from duodena of D-Ca- animals exposed to a single 120 nmol/kg i.v. dose of $1,25(OH)_2D_3$ (Figure 9). The *18S* ribosomal gene was used as a control gene for both the untreated and treated groups. Quantification for the nuclear run-on assays demonstrated that within 6 hours of $1,25(OH)_2D_3$ exposure, the transcription rate of the gene encoding *CYP27A* decreased by 32% when compared to the level of expression observed in control duodena. Non-specific hybridization, estimated by hybridization to pBS plasmid DNA, did not account for the observed *CYP27A* decrease in transcription.

DISCUSSION

Although several studies have reported that the mitochondrial cytochrome P-450 27A is present in multiple sites (14,31,33,37), no study has addressed the regulation of the enzyme in intestine, and the effect of the vitamin D_3 status on the expression of the gene encoding *CYP27A* in small intestine is also unknown. Indeed, most studies to date have focussed on the hepatic regulation of the gene which has, up to now, been reported to be sensitive to bile acids, glucocorticoids, growth hormone and insulin (37,46,48,52,53,55). Axén *et al.* (7), on the other hand, have reported that *CYP27A* located in kidney and liver was affected by $1,25(OH)_2D_3$ administration stressing that the kidney *CYP27A* mRNA levels were decreased to a greater extent than those of the liver, an observation which we have confirmed in our laboratory (51). The significance of these observations on the C-25 hydroxylation of D_3 have not yet been investigated although earlier studies have raised the hypothesis that $1,25(OH)_2D_3$ might inhibit the production of $25OHD_3$ in human subjects (8). Later studies in the rat indicated, however, that the decrease in serum $25OHD_3$ concentrations could mainly be explained by an acceleration in its metabolic clearance rate (15,27,28) although species differences in the C-25 hydroxylation of D_3 have not been ruled out, the microsomal D_3-25 hydroxylase being the predominant enzyme in the rat while, in humans, the mitochondrial CYP27A has been claimed to be the sole D_3-25 hydroxylase (44,45). Our studies show that duodenal *CYP27A* mRNA levels are significantly lower than those observed in liver but, as in liver (55), the duodenal transcript was shown to be significantly upregulated by dexamethasone while common cytochrome P-450 inducers were shown to be without effect. Data also indicate that when compared to duodena obtained from normal D replete animals, *CYP27A* mRNA levels are significantly higher in duodena obtained from D depleted and that, independently of the circulating ionized calcium, clearly indicating that the intestinal *CYP27A* is highly sensitive to the vitamin D status.

Our studies on the effect of D_3, $25OHD_3$ and $1,25(OH)_2D_3$ indicate that each compound significantly lowered the abundance of the *CYP27A* gene transcript. A time-course of the decrease in *CYP27A* mRNA levels following continuous i.p. administration indicates that the decline in mRNA abundance is gradual with significant decreases observed after 72 hours

of $25OHD_3$ repletion and as soon as 24 hours following the initiation of $1,25(OH)_2D_3$ repletion. CYP27A protein levels and 25-hydroxylase enzyme activity were also shown to be sensitive to $1,25(OH)_2D_3$. Furthermore, the studies with the i.v. injection of $1,25(OH)_2D_3$ as well as those on the half-life of *CYP27A* mRNA indicate that the gene transcript is rapidly and dose-dependently down-regulated within hours of exposure to the hormone. These data demonstrate that in rat duodena the gene encoding *CYP27A* is highly sensitive to the *in vivo* exposure to the vitamin D_3 hormone. Whether $1,25(OH)_2D_3$ is the sole mediator of the observed down regulation of the *CYP27A* gene transcript is not known. Attempts at evaluating the specific role of $1,25(OH)_2D_3$ versus that of $25OHD_3$ in the regulation of *CYP27A* gene expression were done using ketoconazole to inhibit the C-1α hydroxylase when $25OHD_3$ was administered. Unfortunately, these attempts were unsuccessful for the following reasons: *i*) ketoconazole proved to be a non-specific monooxygenase inhibitor, inhibiting not only the C-1α (29) but also the C-24 hydroxylase (32,57) which resulted in unexpected changes in circulating $1,25(OH)_2D_3$, *ii*) the effect of $1,25(OH)_2D_3$ on the *CYP27A* gene transcript proved to be too sensitive to evaluate subtle changes in the $1,25(OH)_2D_3$ circulating concentrations induced by ketoconazole, and *iii*) $1,25(OH)_2D_3$ exhibited a rapid effect (significant inhibition observed within 6 hours after i.v. $1,25(OH)_2D_3$ exposure) to discriminate its role versus that of $25OHD_3$ although the time-course of inhibition suggests that the latter is most likely not responsible for the inhibition observed during the present studies. Although the data obtained during our studies suggest a highly significant effect of $1,25(OH)_2D_3$ on the down-regulation of the duodenal *CYP27A* transcript, they do not entirely rule out the participation of other metabolites such as $24,25(OH)_2D_3$, and/or of down stream products of the hormone.

The differences observed in the sensitivity of *CYP27A* between animals repleted with D_3, $25OHD_3$, or $1,25(OH)_2D_3$ warrants comments. Interestingly, an earlier study carried out in our laboratory has indicated that the kinetics of the serum $25OHD_3$ and $1,25(OH)_2D_3$ achieved as well as that of the involution of the associated secondary hyperparathyroidism was quite different when animals were repleted with D_3, or with $1,25(OH)_2D_3$ alone (23). Indeed, the serum $1,25(OH)_2D_3$ concentrations achieved with D_3 repletion are much higher (in the 1500 to 2000 pmol/L range, most likely due to the high 1α-hydroxylase activity

induced by D depletion) than those achieved when the hormone is applied by i.p. mini-osmotic pump which, in the present study, proved to be quite constant averaging 500 to 600 pmol/L between day 1 and day 7 of $1,25(OH)_2D_3$ repletion. These differences could explain the greater effect of the parent compound (after 7 days of repletion) on the steady-state expression of the gene encoding *CYP27A* when compared to that observed following $1,25(OH)_2D_3$ administration. However, the *in vivo* effect of $1,25(OH)_2D_3$ on *CYP27A* mRNA levels is clearly illustrated by the clear dose-response curve achieved following i.v. administration. A clear effect of $1,25(OH)_2D_3$ on *CYP27A* mRNA half-life, and on the transcription rate of the gene also supports an action mediated by the hormone, or by immediate and rapidly formed down-stream metabolite(s).

The data illustrating that the duodenum (as well as the jejunum, ileum and colon) clearly expresses *CYP27A* as well as the D_3-25 hydroxylase protein lead us to put forward the hypothesis that a local production of $25OHD_3$ can be achieved in the small intestine. In addition, the already reported presence of $25OHD_3$-1α hydroxylase and $25OHD_3$-24 hydroxylase activities in intestinal cells as well as in the Caco-2 cell line (5,16,36,47) suggests that D of dietary origin could be locally processed and transformed into 25OHD, $1,25(OH)_2D$, or $24,25(OH)_2D/1,24,25(OH)_3D$. Moreover, Axén *et al.* (6) have reported that the C-27 hydroxylase purified from pig and rabbit livers as well as recombinant human *CYP27A* was also able to catalyse the 1α-hydroxylation of $25OHD_3$ albeit at a much lower rate than that observed for the conversion of D_3 into $25OHD_3$ (7). Furthermore, CYP27A has also been shown to be active on other D compounds such as D_2, and $1\alpha OHD_3$ as illustrated in the present studies (19,20,26). Collectively these observations illustrate that a large spectrum of compounds of the D family can be locally activated by intestinal cells into active metabolites when taken orally.

The critical elements involved in the $1,25(OH)_2D_3$-mediated down regulation of the *CYP27A* gene transcript have not been investigated. Our data indicate that the mechanisms responsible for the regulation of the gene involve a decrease in mRNA half-life and a decrease in transcriptional rate. The effect of calcitriol on the gene, however, is present even in the absence of normalization of the circulating Ca^{2+} concentrations as illustrated following the i.v. injection of $1,25(OH)_2D_3$ suggesting that Ca^{2+} is not a critical element in the response

to the hormone. In addition, repletion with calcium alone (which normalizes the serum Ca^{2+} concentration without affecting the D nutritional or hormonal status) does not affect *CYP27A* steady state mRNA levels. *CYP27A* and *CYP7α* are genes involved in bile acid biosynthesis. *CYP7α* is known to be regulated by some of the orphan receptors mediating the response to fatty acid and cholesterol such as PXR (a nuclear receptor closely related to VDR) (34), FXR or PPAR which all have RXR as partner for DNA binding. It is not yet known, however, whether *CYP27A* is also regulated by these nuclear receptors and/or what is the role of $1,25(OH)_2D_3$ in these interactions most particularly in relation with its binding to the VDR and the subsequent involvement of RXR for DNA binding and activation.

The data obtained during our studies clearly show that the rat duodenum expresses the mitochondrial D_3-25 hydroxylase *CYP27A*. They also show an effect of the D_3 nutritional status (D_3 and $25OHD_3$) as well as of the D_3 hormonal status ($1,25(OH)_2D_3$) on the gene mRNA half-life and transcription rate. Thus, in addition to exhibiting high amounts of VDR, the intestine seems to possess the major D_3 hydroxylases, indicating that aside from being able to respond to the classic endocrine actions mediated by $1,25(OH)_2D_3$, the small intestine may exhibit the presence of a fine intra/paracrine, or autocrine regulation of D_3-related pathways.

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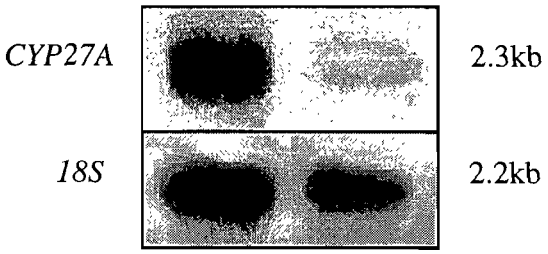
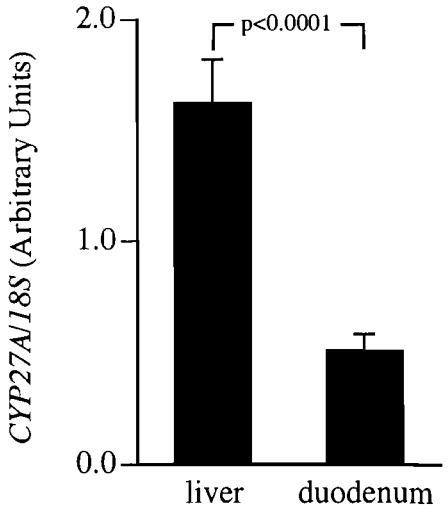
LEGEND TO FIGURE 1

Northern analysis representing the relative abundance of the *CYP27A* gene transcript in male liver and duodenum (A). Means ± S. E. M. *CYP27A* mRNA levels observed in 4 animals/group. Statistically significant differences between group means were analysed by the Student's "t" test.

A


CYP27A
2.3kb
18S
2.2kb

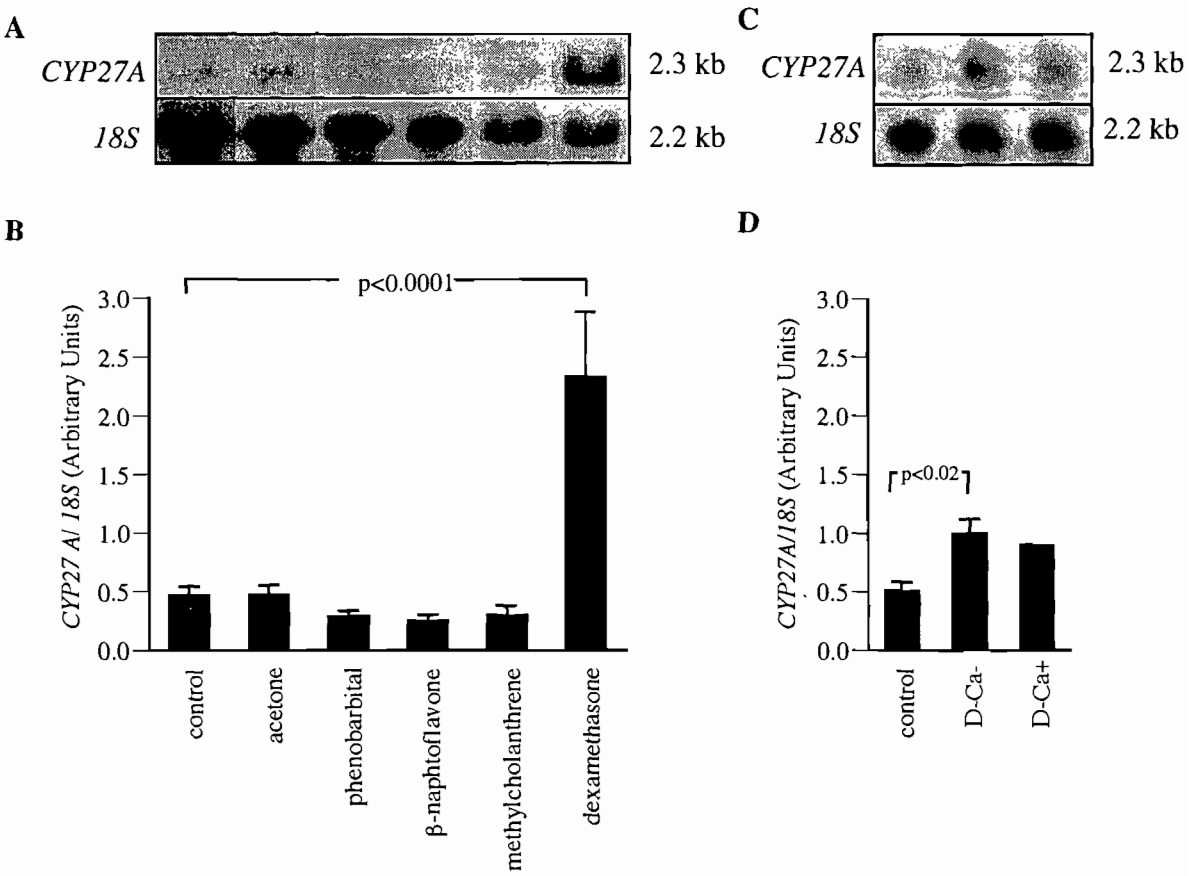
B


2.0
1.0
0.0
p<0.0001
CYP27A/18S (Arbitrary Units)
liver
duodenum

LEGEND TO FIGURE 2

Influence of cytochrome P-450 inducers on the duodenal expression of the gene encoding *CYP27A*. A and C. Representative Northern analyses of the *CYP27A* gene transcript. B. Evaluation of *CYP27A* mRNA levels in normal control male rat duodena and after administration of cytochrome P-450 inducers. n = 3 animals/group. D. Evaluation of the relative level of the *CYP27A* gene transcript in normal, and in hypocalcemic (serum Ca^{2+}: 0.78±0.02) and in normocalcemic (serum Ca^{2+}: 1.26±0.02) vitamin D depleted male rat duodena. Data are presented as means ± S. E. M.

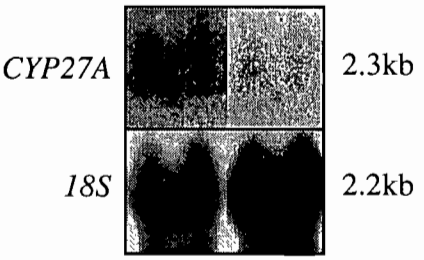
n = 11 for control, n = 15 for D-Ca- and n = 3 for D-Ca+. Statistically significant differences between group means were analysed by ANOVA (cytochrome P-450 induction studies), and by the Student's "t" test (normal and D-Ca- or D-Ca+ studies).


A
CYP27A
18S
2.3 kb
2.2 kb
C
CYP27A
18S
2.3 kb
2.2 kb
B
p<0.0001
CYP27 A/ 18S (Arbitrary Units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
acetone
phenobarbital
β-naphtoflavone
methylcholanthrene
dexamethasone
D
p<0.02
CYP27A/18S (Arbitrary Units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
D-Ca-
D-Ca+

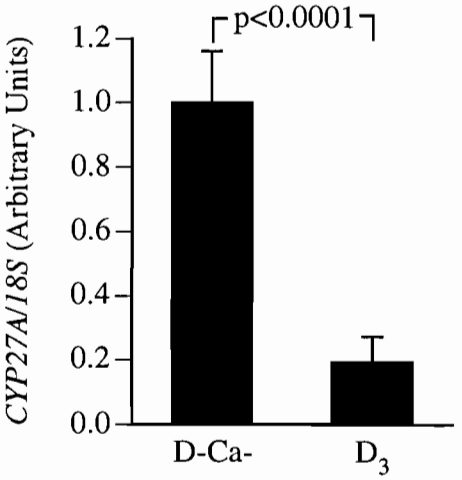
LEGEND TO FIGURE 3

Influence of D_3 repletion on the duodenal expression of the gene encoding *CYP27A*. A. Representative Northern analysis of the *CYP27A* gene transcript in D-Ca-, and after 7 days of D_3 repletion by mini-osmotic pump (i.p.) at a dose of 6.5 nmol/day. B. Steady-state levels of *CYP27A* mRNA in D-Ca-, and in D_3 repleted rat duodena. Data are presented as means ± S. E. M. n = 5 animals/group. Statistically significant differences between group means were analysed by the Student's "t" test.

A


CYP27A
2.3kb
18S
2.2kb

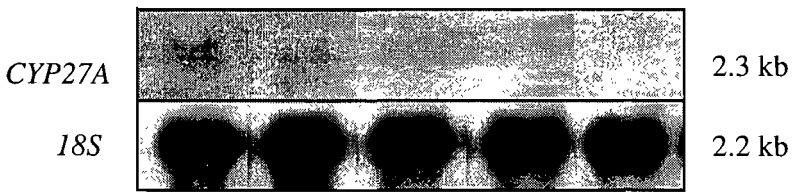
B


p<0.0001
CYP27A/18S (Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
D-Ca-
D3

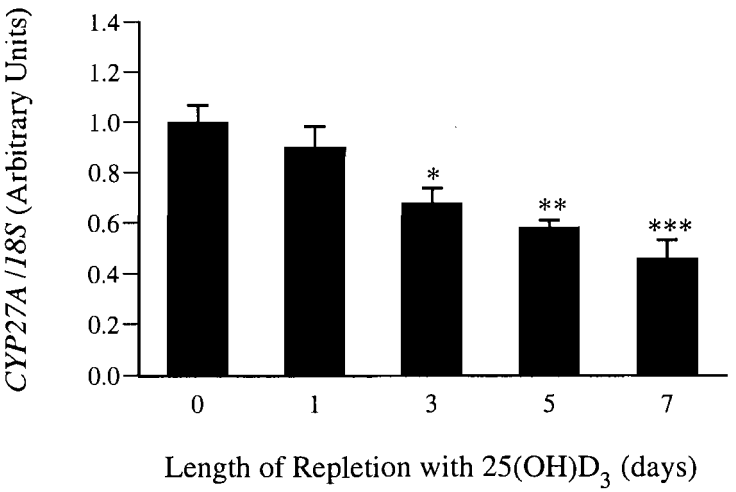
LEGEND TO FIGURE 4

Influence of $25OHD_3$ repletion on the duodenal expression of the gene encoding *CYP27A*. A. Representative Northern analysis of the *CYP27A* gene transcript in D-Ca-, and following 1, 3, 5 or 7 days of $25OHD_3$ repletion by mini-osmotic pump (i.p.) at a dose of 28 pmol/day. B. Steady-state levels of *CYP27A* mRNA in D-Ca-, and in $25OHD_3$ repleted rat duodena. Data are presented as means ± S. E. M. n = 3 animals/group. Statistically significant differences between group means were analysed by ANOVA with individual contrasts evaluated by the Bonferonni test. Main effect, $p<0.001$, significantly different from D-Ca-, * $p<0.003$, ** $p<0.002$, *** $p<0.0008$.

A

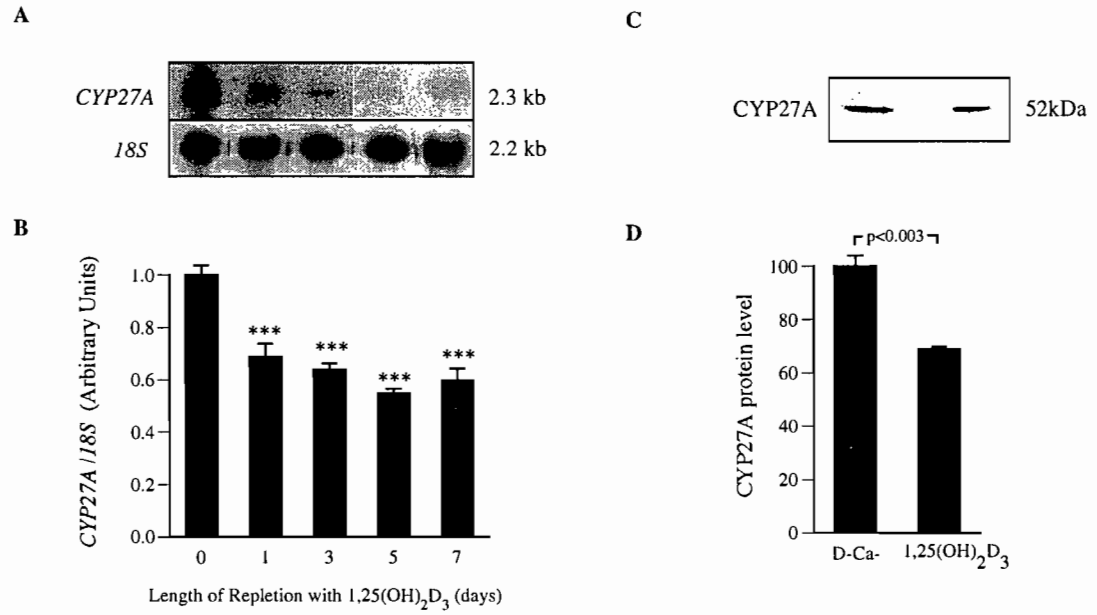

CYP27A
18S
2.3 kb
2.2 kb

B


CYP27A /18S (Arbitrary Units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
***
0
1
3
5
7
Length of Repletion with 25(OH)D3 (days)

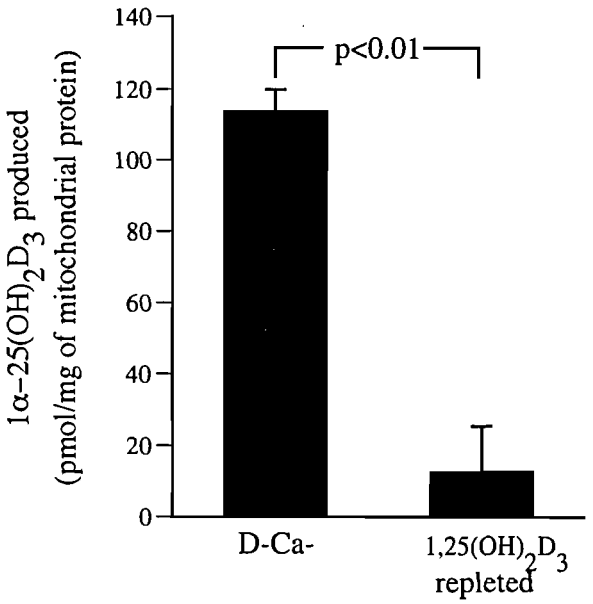
LEGEND TO FIGURE 5

Influence of $1,25(OH)_2D_3$ repletion on the duodenal expression of the gene encoding *CYP27A*, and on the level of the CYP27A protein. A. Representative Northern analysis of the *CYP27A* gene transcript in D-Ca-, and following 1, 3, 5 or 7 days of $1,25(OH)_2D_3$ repletion by mini-osmotic pump (i.p.) at a dose of 28 pmol/day. B. Steady-state level of *CYP27A* mRNA in D-Ca-, and in $1,25(OH)_2D_3$ repleted rat duodena. C. Representative Western analysis of the CYP27A protein in D-Ca-, and 7 days after $1,25(OH)_2D_3$ repletion. D. Steady-state level of CYP27A protein in D-Ca-, and in $1,25(OH)_2D_3$ repleted rat duodena. Data are presented as means ± S. E. M. n = 3 animals/group. Statistically significant differences between group means were analysed by ANOVA with individual contrasts evaluated by the Bonferonni test. Main effect, $p<0.0002$, significantly different from D-Ca-, ***$p<0.0008$.


A
CYP27A
18S
2.3 kb
2.2 kb
B
CYP27A /18S (Arbitrary Units)
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
***
0
1
3
5
7
Length of Repletion with 1,25(OH)2D3 (days)
C
CYP27A
52kDa
D
p<0.003
CYP27A protein level
100
80
60
40
20
0
D-Ca-
1,25(OH)2D3

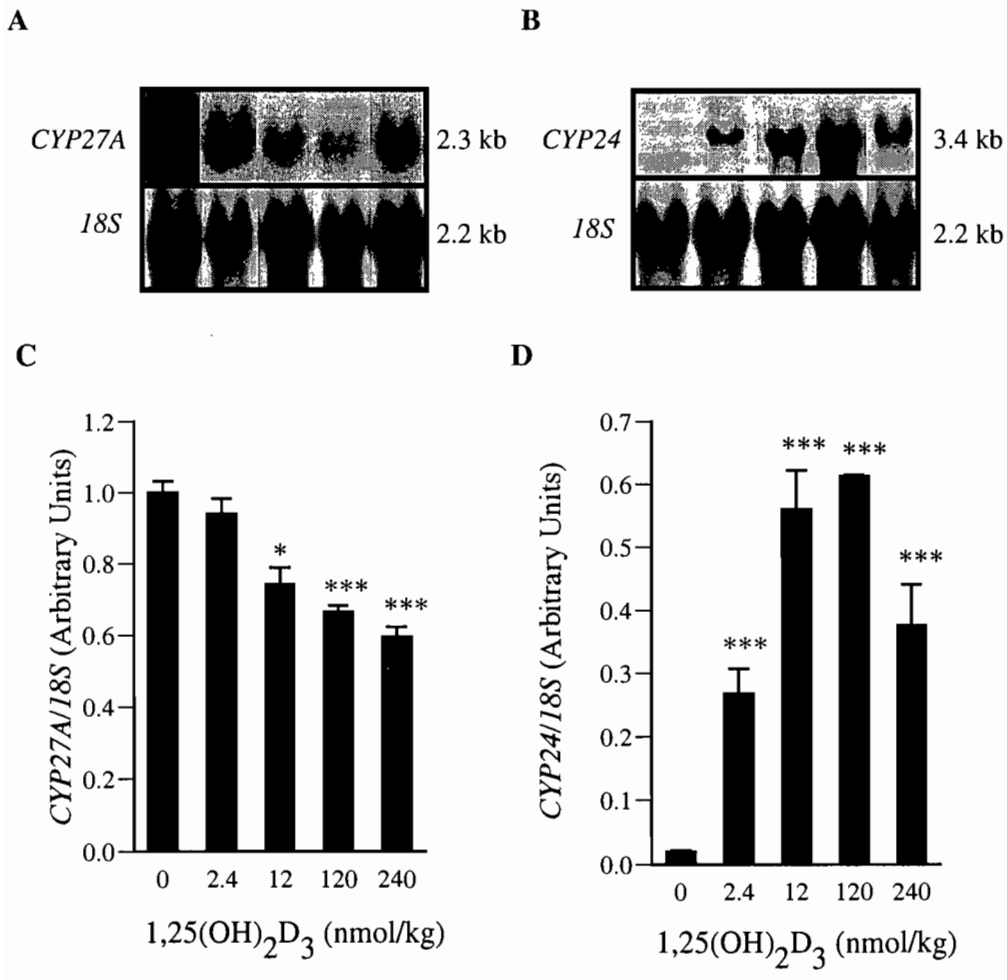
LEGEND TO FIGURE 6

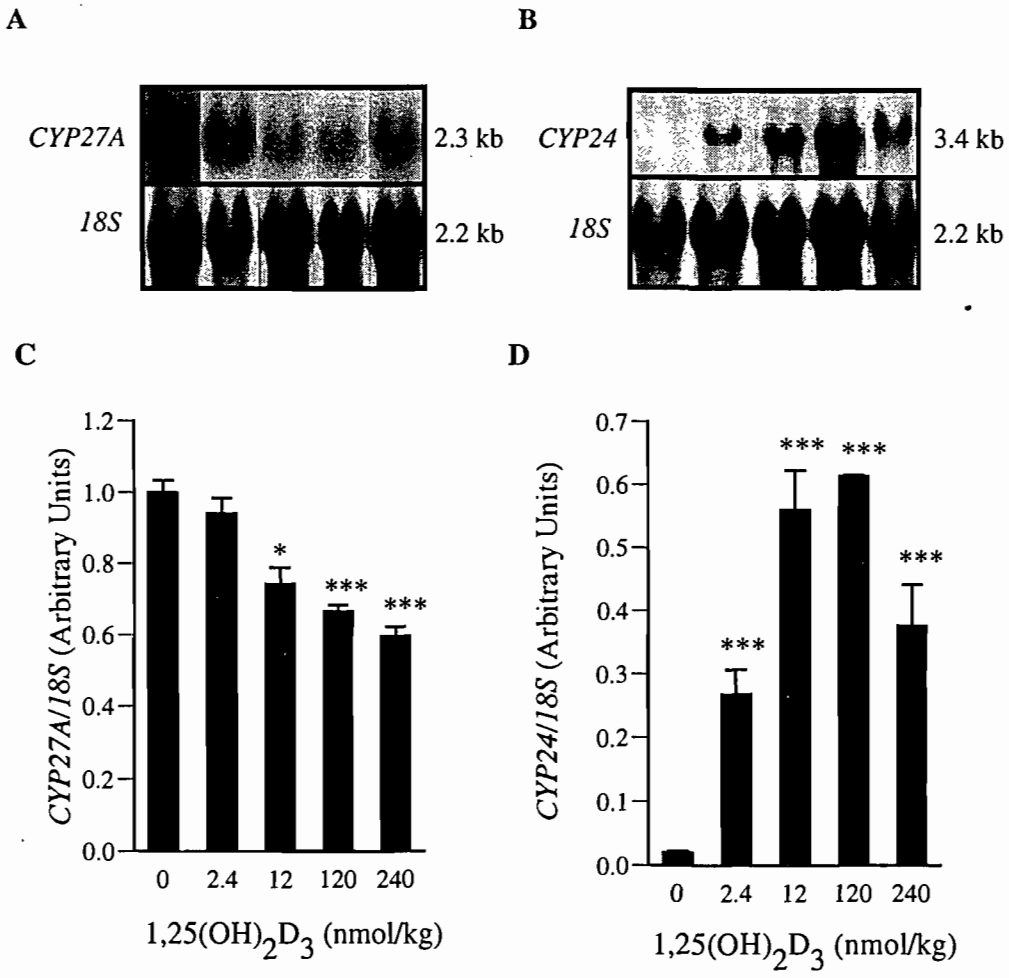
Mitochondrial 25-hydroxylase activity from freshly isolated duodenal mucosal cells obtained from hypocalcemic vitamin D depleted (D-Ca-) (n=2) or $1,25(OH)_2D_3$ repleted rats (n=3). Statistically significant differences between group means were analyzed by the Student's "t" test.


140
120
100
80
60
40
20
0
p<0.01
1α-25(OH)2D3 produced
(pmol/mg of mitochondrial protein)
D-Ca-
1,25(OH)2D3
repleted

LEGEND TO FIGURE 7

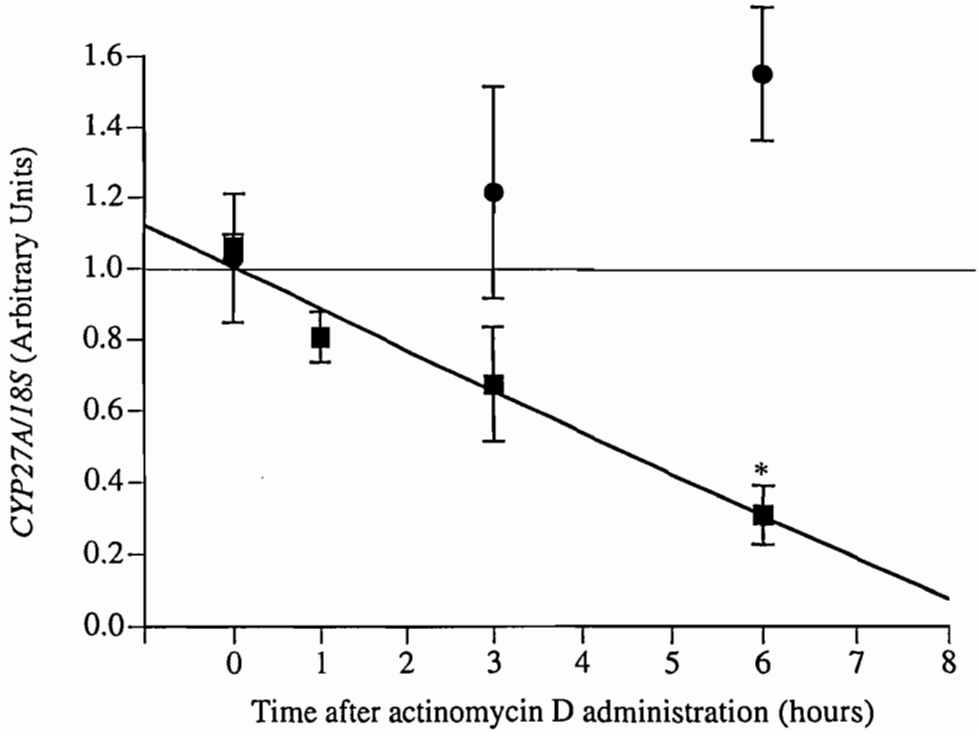
Influence of acute $1,25(OH)_2D_3$ administration on the expression of the genes encoding *CYP27A* and *CYP24*. All animals received a single i.v. dose of 0, 2.4, 12, 120, or 240 nmol/kg and killed 6 hours later. A. Representative Northern analysis of the *CYP27A* gene transcript in D-Ca-, and in animals exposed to $1,25(OH)_2D_3$. B. Steady-state levels of *CYP27A* mRNA in duodena of D-Ca- and of animals injected with $1,25(OH)_2D_3$. C. Representative Northern analysis of the *CYP24* gene transcript in D-Ca-, and in animals exposed to $1,25(OH)_2D_3$. D. Steady-state levels of *CYP24* mRNA in duodena of D-Ca- and of animals injected with $1,25(OH)_2D_3$. n=3 animals/group. Statistically significant differences between group means were analysed by ANOVA with individual contrasts evaluated by the Bonferroni test. *CYP27A* mRNA levels: Main effect: $p<0.003$. Statistically different from D-Ca-, * $p<0.03$, ** $p<0.0008$; *CYP24* mRNA levels: Main effect: $p<0.0001$. Statistically different from D-Ca-, *** $p<0.0008$.


A
CYP27A
2.3 kb
18S
2.2 kb
B
CYP24
3.4 kb
18S
2.2 kb
C
CYP27A/18S (Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
***
***
0
2.4
12
120
240
1,25(OH)2D3 (nmol/kg)
D
CYP24/18S (Arbitrary Units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
***
***
0
2.4
12
120
240
1,25(OH)2D3 (nmol/kg)


A
B
CYP27A
18S
2.3 kb
2.2 kb
CYP24
18S
3.4 kb
2.2 kb
C
D
CYP27A/18S (Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
***
***
0
2.4
12
120
240
1,25(OH)2D3 (nmol/kg)
CYP24/18S (Arbitrary Units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
***
***
***
0
2.4
12
120
240
1,25(OH)2D3 (nmol/kg)

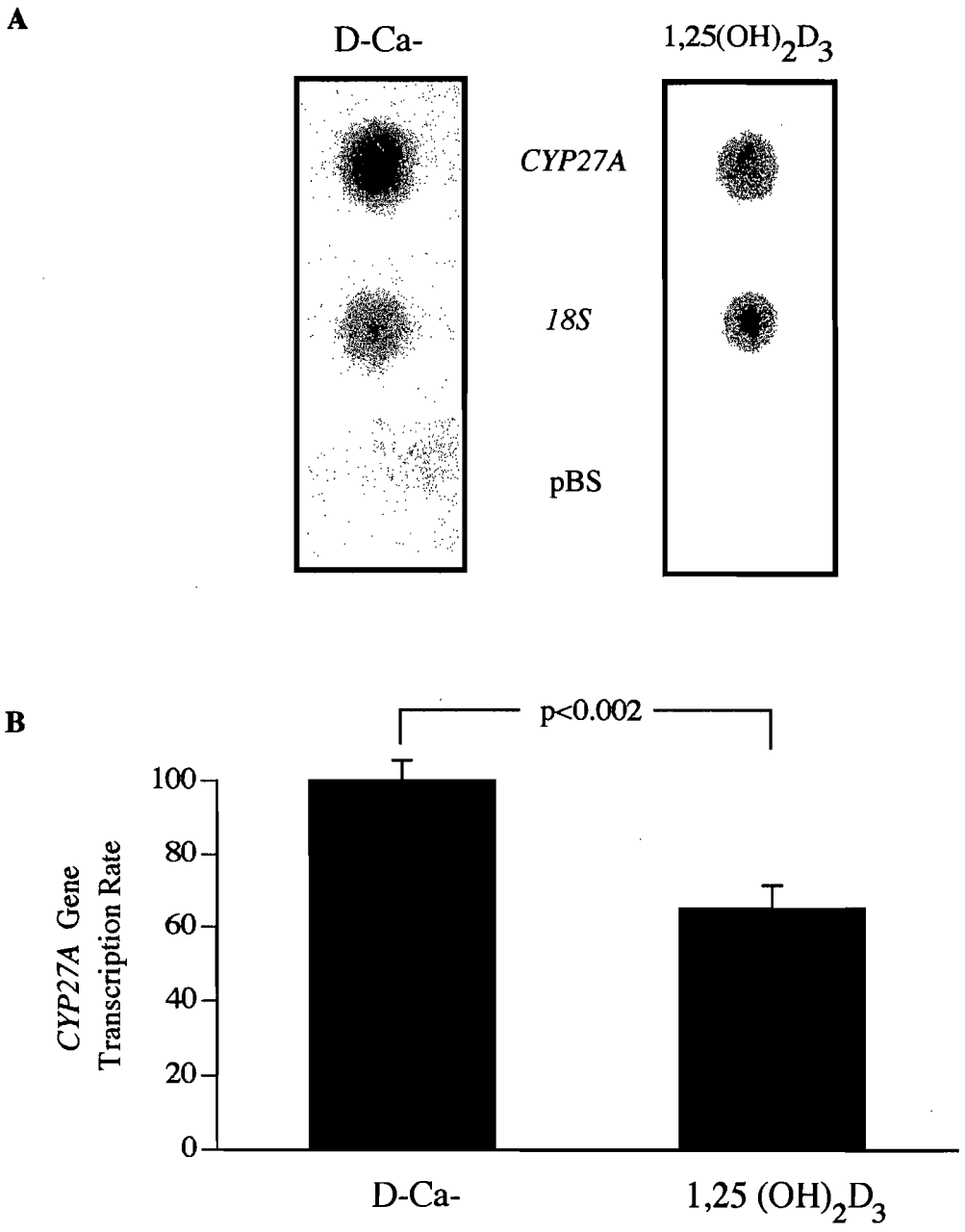
LEGEND TO FIGURE 8

In vivo half-life of *CYP27A* mRNA in duodena of D-Ca- and $1,25(OH)_2D_3$ injected rats. Animals received a single i.v. dose (120nmol/kg) of either $1,25(OH)_2D_3$ or vehicle, 3 hours before the actinomycin D (0.5mg/kg) administration . Animals were killed 1, 3, or 6 hours after actinomycin D administration. ● *CYP27A* mRNA levels in D-Ca- rat duodena, ■ *CYP27A* mRNA levels in $1,25(OH)_2D_3$ injected rat duodena. Data are presented as means ± S. E. M. n = 2 animals/group. Statistically significant differences between group means were analysed by ANOVA, with individual contrasts evaluated by the Bonferonni test. Main effect, $p<0.04$, Significantly different from D-Ca-, * $p<0.03$.


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
0
1
2
3
4
5
6
7
8
CYP27A/18S (Arbitrary Units)
Time after actinomycin D administration (hours)

LEGEND TO FIGURE 9

Rate of transcription of the *CYP27A* gene transcript in duodena of D-Ca- and $1,25(OH)_2D_3$ injected rats. Animals received a single i.v. dose (120nmol/kg) of $1,25(OH)_2D_3$ 6 hours before euthanasia. A. Representative nuclear run-on transcriptional assays. B. Quantitative evaluation of the transcriptional run-on assays was achieved by scanning densitometry. Transcriptional activity was measured in three different experiments using 2-3 rats per experiment for each group. Data are presented as means ± S. E. M. Statistically significant differences between group means were analysed by the Student's "t" test.


A
D-Ca-
1,25(OH)2D3
CYP27A
18S
pBS
B
p<0.002
CYP27A Gene Transcription Rate
100
80
60
40
20
0
D-Ca-
1,25 (OH)2D3

3.2.2 Article 4: Calcitriol regulates the expression of the gene encoding all three vitamin D_3 hydroxylases and the drug metabolizing enzyme CYP3A4 in the human fetal intestine.

Article in press, Journal of Clinical Endocrinology.

(ARTICLE 4)

CALCITRIOL REGULATES THE EXPRESSION OF THE GENES ENCODING THE THREE KEY VITAMIN D_3 HYDROXYLASES AND THE DRUG METABOLIZING ENZYME CYP3A4 IN THE HUMAN FETAL INTESTINE

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Short title: D_3 hydroxylases in the human fetal intestine

Key words: Vitamin D_3 hydroxylases, CYP27A, CYP27B1, CYP24, CYP3A4, VDR, $1,25(OH)_2D_3$, $25OHD_3$, human intestine

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ABSTRACT

The human fetal jejunum has been shown to harbour the vitamin D_3 (D_3) nuclear receptor (VDR_n) and to be responsive to calcitriol/1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) through modulation of proliferation and differentiation processes. The aim of the study was to evaluate the presence as well as the effect of $1,25(OH)_2D_3$ exposure on the expression levels of the three key D_3-hydroxylase gene transcripts (25-hydroxylase, *CYP27A*; 24-hydroxylase, *CYP24*; 1α-hydroxylase, *CYP27B1*) as well as that of the $1,25(OH)_2D_3$-responsive endobiotic/xenobiotic metabolizing enzyme *CYP3A4* (which is also considered a major detoxifiying enzyme) in the human proximal and distal intestine. Specimens from normal fetuses ranging from 15 to 20 weeks of gestation were obtained following elective termination of normal pregnancies. Intestinal explants were cultured for a period of 24h or 48h with 10^{-7}M $1,25(OH)_2D_3$. All data were compared to paired-control cultures without $1,25(OH)_2D_3$. Total RNA was extracted and cDNA synthesized by RT-PCR. The cDNA obtained was amplified by radioactive PCR, the signal intensity evaluated by densitometric analyses and expressed in relation to the levels of *GAPDH*. Data indicate that *VDR_n*, the three D_3-hydroxylases as well as *CYP3A4* are expressed in all segments of the human fetal small intestine and in the colon. Basal expression levels of *VDR_n*, *CYP27A*, *CYP24* and *CYP3A4* were found to be similar in the proximal, median and distal jejunum as well an in the proximal and distal colon. In contrast, basal 1α-hydroxylase *CYP27B1* expression levels were found to be 65% higher in the colon than in the small intestine ($p<0.02$). The 1α-hydroxylase was also found to be sensitive to $1,25(OH)_2D_3$ with a 31% decrease in its expression levels within 24h of $1,25(OH)_2D_3$ exposure to reach a 55% decrease after 48h of incubation in the presence of

the hormone ($p<0.05$). Furthermore, the levels of the 25-hydroxylase gene transcript were also decreased by 10% within the first 24h and by 29% after 48h of incubation in the presence of $1,25(OH)_2D_3$ ($p<0.003$). *VDR_n* expression levels were also found to be reduced following incubation in the presence of $1,25(OH)_2D_3$. In contrast, exposure to $1,25(OH)_2D_3$ contributed to a 4.8 fold increase in the expression of the 24-hydroxylase gene transcript within the first 24h of exposure ($p<0.03$), and to a highly significant induction (24, 22 and 1.5 fold over basal values) of the *CYP3A4* gene transcript in 3 of the 4 specimens studies. Collectively, the data illustrate that at mid-gestation $1,25(OH)_2D_3$ is fully active in the modulation of all D_3-hydroxylases in the human developing intestine. They also show that the detoxifying enzyme *CYP3A4* is not only present along the intestinal tract but is also sensitive to $1,25(OH)_2D_3$ indicating that the hormone may be a key element in intestinal development and in the maintenance of the intestinal mucosa integrity in the basal state and in response to damage-inducing agents.

INTRODUCTION

Pregnancy leads to several changes in maternal calcium homeostasis including significant increases in the circulating concentrations of the vitamin D (D/D_3) binding protein (DBP) (Haddad, Jr. & Walgate 1976; Abbas *et al.*, 1987) as well as in the total and free serum concentrations of the main D_3 metabolites 25-hydroxyvitamin D_3 ($25OHD_3$) and 1,25-dihydroxyvitamin D_3 (calcitriol, $1,25(OH)_2D_3$) (Kumar *et al.*, 1979; Bouillon *et al.*, 1981; Bikle *et al.*, 1984; Weiss *et al.*, 1998). These adjustments contribute to adequately meet the large calcium and phosphate requirements of the growing fetus and to preserve the maternal skeleton by increasing intestinal calcium and phosphate absorption in the mother (Christakos & Norman 1980; Halloran & DeLuca 1980; Care, 1997).

In the last three decades, several groups have reported the presence of a positive relationship between the maternal and the fetal or venous umbilical cord blood $25OHD_3$ as well as between the maternal and fetal $1,25(OH)_2D_3$ concentrations in humans (Bishop & Salle, 1997; Rummens *et al.*, 2000). In addition, in all species studied, D_3 metabolites have been shown to be transferred across the placenta (Haddad, Jr. *et al.*, 1971; Hillman & Haddad, 1974; Bouillon *et al.*, 1981; Goff *et al.*, 1982; Ron *et al.*, 1984). However, despite positive correlations between the mother and the fetus, circulating D_3 metabolites have consistently been reported to be lower in the fetus than in the mother (Weisman *et al.*, 1978; Gertner *et al.*, 1980; Paulson *et al.*, 1987). These observations have led to the hypothesis that the fetus is dependent on the mother to satisfy its needs in $25OHD_3$ and in $1,25(OH)_2D_3$ (Bishop & Salle, 1997; Salle *et al.*, 2000). This is further illustrated by the presence of rickets in infants born of mothers presenting signs of D deficiency (Ford *et*

al., 1973; Park *et al.*, 1987).

Despite the reported dependence of the fetus on the mother's D supply, the presence of enzymes involved in D metabolism have been reported in several fetal organs. Indeed, the mitochondrial D_3 25-hydroxylase gene transcript has been shown to be expressed in the liver and kidney of 17 to 19 week-old human fetuses (Gascon-Barré *et al.*, 2001) while expression of the 1α-hydroxylase has been reported in the kidney, intestine and bone of mouse fetuses (Ishida *et al.*, 1988; Panda *et al.*, 2001) indicating that the enzyme is not only present in the kidney but also in "non-classical" tissues and organs as is the case in human adult subjects (Zehnder *et al.*, 2001). Moreover, Wieland *et al.* (Wieland *et al.*, 1980) have proposed, based on higher $1,25(OH)_2D_3$ concentrations in fetal arterial blood compared to those observed in umbilical vein, that the human fetal kidney was able of synthesizing $1,25(OH)_2D_3$. In support of the latter hypothesis, Moore *et al.* (Moore *et al.*, 1985) have shown that fetal circulating $1,25(OH)_2D_3$ concentrations, although decreased, were still detectable when pregnant sheep were bilaterally nephrectomized thus clearly indicating that the fetus is able to synthesize the D_3 hormone (Moore *et al.*, 1985).

The aim of the present studies was to *i)* investigate the presence of the genes encoding the three key D_3 metabolizing enzymes, the mitochondrial 25- (*CYP27A*), 24- (*CYP24*), and 1α-(*CYP27B1*) hydroxylases, the nuclear D_3 receptor (VDR_n) as well as a recently identified $1,25(OH)_2D_3$-responsive gene, cytochrome P450 3A4 (*CYP3A4*) in human fetal jejunum and colon, and *ii)* study the effect of $1,25(OH)_2D_3$ exposure *in vitro* on their transcript levels in the proximal intestine.

MATERIALS AND METHODS

CHEMICALS

Garamycin and serum-free Leibovitz L-15 medium were obtained from Gibco BRL (Burlington, ON, Canada), One-Step RT-PCR from Qiagen (Mississauga, ON, Canada), [α-^{32}P] dCTP from ICN Biomedicals Inc. (Mississauga, ON, Canada), Kodak NBT-2, D19 developer and fixer from Interscience (Mississauga, ON, Canada). 1,25$(OH)_2D_3$ was a gift from Hoffmann-LaRoche Ltd. (Mississauga, ON, Canada).

SPECIMENS

Small intestine (proximal, median and distal jejunum), colon (proximal, distal) as well as liver and kidney specimens from normal fetuses 15-20 weeks of postfertilization age (4-5 subjects for each condition studied) were obtained following voluntary termination of normal pregnancy. No tissue was collected from cases associated with known fetal abnormality or fetal death. All intestinal segments were isolated according to anatomical location. The specimens were immersed in Leibovitz L-15 medium (room temperature) containing Garamycin (40mg/ml) and brought to room temperature within 30min. The tissues were frozen in liquid nitrogen and kept at -80°C until further processing for PCR analyses.

Normal liver and kidney specimens were also obtained from adult subjects recruited among patients referred for hepatic or renal surgery at St-Luc Hospital, Montreal, Canada (4 men and one woman, mean age 63 ± 2.8).

Protocols were approved by the Institutional Ethics Review Board and informed consents were obtained from all women before voluntary termination of pregnancy as

well as from all adult volunteers.

ORGAN CULTURE

The small intestine of each fetus was cleansed of mesentery, split longitudinally, washed in culture medium and cut into several explants (3X7mm). Explants were randomly transferred onto lens paper in each organ culture dish (Falcon Plastics, Los Angeles, CA) and cultured in serum-free Leibovitz L-15 medium according to the technique described earlier (Ménard *et al.*, 1988). After a 3-hour stabilization period, $1,25(OH)_2D_3$ in ethanol was added to the culture medium at a final concentration of 10^{-7}M, while control specimens were exposed to the vehicle only. Proximal intestinal explants were cultured for periods of 24h and 48h. The tissues were removed from the culture medium and immediately snap frozen in liquid nitrogen and kept at -80°C until ready for PCR analyses.

SAMPLE EXTRACTION AND RT-PCR ANALYSES

Total intestinal RNA was extracted as previously described (Lemay *et al.*, 1995). After Dnase treatment, 1 µg of total RNA was converted in cDNA (First-Strand cDNA synthesis Kit) using $pd(N)_6$ as primer; 2,0 µL of RT reaction was amplified for either 25 (*CYP27A*), 35 (*CYP27B1*), 30 (*CYP24*), 27 (*VDR*), 30 (*CYP3A4*) and 20 cycles (*GAPDH*) using specific primers and Taq PCR Master Mix and 0,1 µL of [α-^{32}P] dCTP (3 000 Ci/mol) in a Touchdown Thermal Cycling system (Hybaid, Teddington, U.K.). Design of primers to generate *CYP27A*, *CYP27B1*, *CYP24*, *VDR* and *GAPDH* cDNA fragments was made with the Primers Software of Williamstone Enterprises (http://www.williamstone.com) from the sequences of Cali *et al.* (Cali *et al.*, 1991),

(*CYP27A*) Fu *et al.* (Fu *et al.*, 1997) (CYP27B1), Chen *et al.* (Chen *et al.*, 1993) (*CYP24*), Baker *et al.* (Baker *et al.*, 1988) (*VDR*) and Tso *et al.* (Tso *et al.*, 1985) (*GAPDH*). *CYP3A4* primers were identical to those used by Schmiedlin-Ren P *et al.* (Schmiedlin-Ren *et al.*, 2001) (Table 1). cDNA PCR products were loaded and separated onto a non-denaturing 8% polyacrylamide TBE gel. The gel was dried and exposed to Kodak X-Omat AR film at -80°C in the presence of an intensifying screen for 4 to 16 hours. Densitometry was performed as described previously (Lemay *et al.*, 1995; Demers *et al.*, 1997). All values are reported in relation to the level of expression of the housekeeping gene *GAPDH* for each sample analyzed. The number of PCR cycles for each gene was in the linear portion of the cDNA amplicons generated as indicated in the figure legends.

Statistical Analysis

Several RT-PCR replicates were obtained for each specimen and the mean value obtained. For each condition, the group mean ± SEM was then obtained using the mean replicate value of each condition. Data obtained following $1,25(OH)_2D_3$ exposure are presented as mean ± SEM % values obtained in comparison to paired-control specimens incubated in the absence of $1,25(OH)_2D_3$. The mean levels of expression in the fetal jejunum and in the colon were calculated from the levels of expression observed in all jejunum or colon segments of all individual specimens studied. Statistical significant differences between group means were determined by the Student's "t" test for paired and unpaired variates as indicated in the figure legends.

RESULTS

The D_3 25-hydroxylase *CYP27A*

The representative expression profile of the *CYP27A* gene transcript in the proximal, median and distal jejunum as well as in the proximal and distal colon of specimens obtained from fetuses of 15-20 weeks gestation is presented in Figure 1A. As illustrated, *CYP27A* is clearly present in all segments of the fetal intestine and although higher in the colon than in the jejunum, no significant differences were found in the mean steady state expression levels of the *CYP27A* gene transcript between the small and the large intestine (Fig. 1A and B). However, when compared to the fetal or adult liver (Fig. 1C and D) (the main expression site of the *CYP27A* gene transcript), the fetal jejunum expression levels of the *CYP27A* gene were found to be two fold lower than those found in fetal liver ($p<0.0004$), and 5.9 fold lower than those found in adult livers ($p<0.0001$). The differences observed between the fetal or adult liver and the fetal colon were 1.5 fold (N.S.) and 4.5 fold ($p<0.0001$) lower in the colon than in the fetal or adult liver respectively.

The effect of $1,25(OH)_2D_3$ exposure *in vitro* on *CYP27A* expression levels is presented in Figure 2. As indicated, incubation in the presence of $1,25(OH)_2D_3$ for a period of 24h led to a 10% decrease in *CYP27A* mRNA levels (N.S.) while a further 19% decrease was observed after 48h of $1,25(OH)_2D_3$ exposure to reach a level of expression of 71% compared to specimens not exposed to $1,25(OH)_2D_3$ ($p<0.003$).

The D_3 1α-hydroxylase *CYP27B1*

Figure 3A presents a representative expression profile of the *CYP27B1* gene

transcript in the fetal proximal, median and distal jejunum as well as in the fetal proximal and distal colon. As illustrated, *CYP27B1* was found to be present in all segments of the fetal intestine. Moreover, calculation of the mean level of expression of the gene in the fetal jejunum and in the colon (Fig. 3B) revealed a 65% higher *CYP27B1* mRNA levels in the colon than in the jejunum ($p<0.02$). Comparisons between the intestinal and renal *CYP27B1* mean expression levels (the main expression site of the gene encoding *CYP27B1* (Fig. 3C and D) indicate that the fetal small intestine *CYP27B1* mRNA levels were 20% lower than those found in fetal kidney (N.S.) and 32 fold lower than those found in the adult kidney ($p<0.0001$). Comparisons between the mean *CYP27B1* mRNA levels found in the fetal colon and those found in the kidney indicate that the fetal colon *CYP27B1* levels were similar to those found in the fetal kidney but 19.5 fold lower than those found in the adult kidney ($p<0.0001$).

Figure 4 presents the effect of $1,25(OH)_2D_3$ exposure on the expression of the *CYP27B1* gene transcript. As indicated, after 24h exposure to $1,25(OH)_2D_3$ (Fig. 4), *CYP27B1* mRNA levels were found to decreased by 31% (N.S.) compared to the levels found in specimens not exposed to the hormone. After 48h incubation in the presence of the hormone, the mean level of expression of the *CYP27B1* gene was found to represent only 45% of those observed in control specimens ($p<0.05$).

The D_3 24-hydroxylase *CYP24* and drug-metabolizing enzyme *CYP3A4*

Figure 5A illustrates the representative expression profile of the *CYP24* gene transcript in the various segment of the jejunum and the colon. As illustrated (Fig. 5A and B), similar expression levels of the *CYP24* gene transcript were found along the intestinal tract with no significant differences observed in mean *CYP24* levels between the jejunum

and the colon.

CYP3A4 expression levels were investigated in four small intestinal and colon specimens. Basal expression of the *CYP3A4* gene transcript was clearly found in all specimens studied as illustrated in Figure 5C and D with no observed differences in mRNA levels between the jejunum and the colon.

Studies on the effect of $1,25(OH)_2D_3$ on the mean *CYP24* mRNA levels (Fig. 6A) revealed that the gene expression levels were significantly upregulated within the first 24h of incubation in the presence of the hormone with an observed mean 4.8-fold increase compared to the values observed in control specimens ($p<0.03$). A 2-fold increase was also observed after 48h of incubation in the presence of $1,25(OH)_2D_3$ as illustrated in Figure 6A ($p<0.07$).

Similarly, exposure to $1,25(OH)_2D_3$, led to a 1.5-, 22- and 24-fold induction respectively in *CYP3A4* mRNA levels in three of the specimens studied (Fig. 6B) while in one specimen *CYP3A4* mRNA levels decreased by 90% compared to its paired control not exposed to $1,25(OH)_2D_3$. Calculation of the Pearson's correlation coefficient between *CYP24* and *CYP3A4* mRNA levels following exposure (Fig. 6C) illustrates a positive relationship between these two VDR_n-regulated genes ($p<0.03$).

The nuclear D receptor *VDR_n*

Figure 7A and B presents the steady state expression levels of the gene encoding VDR_n. As illustrated, VDR_n mRNA was found in all segments of the small and large intestine. No significant differences were found between the mean *VDR_n* mRNA steady state expression levels in the jejunum and the colon. When compared with the fetal or adult kidney (Fig. 7C), the fetal jejunum and colon were found to exhibit lower mRNA

levels than both the fetal or adult kidney respectively (N.S.).

As illustrated in Figure 7D, VDR_n mRNA levels were significantly downregulated within 24h of incubation in the presence of $1,25(OH)_2D_3$ ($p<0.005$). Values remained within the same expression levels after 48h of incubation in the presence of the hormone ($p<0.03$).

DISCUSSION

Our studies clearly illustrate, for the first time, the presence of the genes encoding all three key D_3 hydroxylases (25-hydroxylase *CYP27A*, 1α-hydroxylase *CYP27B1*, and 24-hydroxylase *CYP24*) as well as *CYP3A4* in both the small and large intestine of human fetuses aged 15-20 weeks. Moreover, in accordance with previous studies by Delvin and Ménard (Ménard *et al.*, 1995; Delvin *et al.*, 1996), the VDR_n was also clearly found in both the jejunum and the colon. To date, however, little is known about the regulation of the human fetal intestinal endobiotic/xenobiotic metabolizing enzymes, although metabolic activities and the expression of several cytochrome P-450s have been reported in both animal and human fetal intestine where biotransformation activities are generally found to be greater in the proximal intestine than in the ileum and colon (Traber *et al.*, 1988; Flinois *et al.*, 1992; Toda *et al.*, 1994; Dai *et al.*, 2001). Comparison of the D_3 metabolizing enzymes along the intestinal tract revealed that only the 1α-hydroxylase exhibited a higher expression level in the colon than in the small intestine. In addition, the steady-state 1α-hydroxylase mRNA levels observed in the fetal jejunum and colon were similar to those found in the fetal kidney, the main expression site of the enzyme after birth. These observations indicate a very early acquisition of the D_3 1α-hydroxylase gene in the intestine where $1,25(OH)_2D_3$ has been shown to be an essential hormonal

factor for the complex regulation of human gut development (Arsenault & Ménard, 1987; Ménard *et al.*, 1995). These observations suggest that, during fetal life, the auto/paracrine action of the hormone may be more important than its endocrine action which involves the classical Ca^{2+}-PTH-kidney loop with the hormone mainly acting at distal sites.

Our studies also illustrate that all genes studied were influenced by $1,25(OH)_2D_3$. Indeed, the hormone clearly upregulated expression of the D_3 24-hydroxylase gene (a prototype gene for its response to $1,25(OH)_2D_3$) by close to five-fold at the 24h time-point following incubation in the presence of the hormone while a two-fold induction was still present at the 48h time-point. The decrease in the induction profile at the 48h time-point compared to that observed earlier may be due to a decreased sensitivity of the $1,25(OH)_2D_3$-mediated response after prolonged exposure to the hormone. Interestingly, *CYP3A4*, which has recently been shown to be a $1,25(OH)_2D_3$-responsive gene (Thummel *et al.*, 2001) and a VDR_n-mediated lithocholic acid sensor (Makishima *et al.*, 2002), was not only clearly identified in the small intestine but also in the colon with no observed differences between the two sites. This observation is interesting since in the adult intestine, the expression of the gene has been reported to be higher in the small intestine than in the colon (De Waziers *et al.*, 1989; Paine *et al.*, 1997). Our data, thus, indicate that in the developing human intestine, both intestinal segments seem to constitutionally express similar levels of the *CYP3A4* gene transcript.

Most interestingly, our studies are the first to report a $1,25(OH)_2D_3$-mediated regulation of the gene encoding *CYP3A4* in the human intestine. Indeed, *CYP3A4* was shown to be highly inducible by $1,25(OH)_2D_3$ (over 2000% over basal values in two of the specimens studied) while a more modest, but nevertheless important induction (150% over basal value) was observed in a third specimen. No induction was observed in the

forth specimen studied. The large heterogeneity in the induction of the *CYP3A4* gene transcript may be due to the known genetically determined inter-individual variability in the constitutive expression of the gene (Guengerich, 1999; Ozdemir *et al.*, 2000), as well as in variability in several factors known to be involved in its induction. Indeed, several endogenous and exogenous ligands acting through the pregnane X receptor/steroid and xenobiotic receptor (PXR/SXR) are known to induce *CYP3A4* (Honkakoski & Negishi, 2000) while the induction of *CYP3A4* by $1,25(OH)_2D_3$ has been shown to be mediated by both VDRE and PXRE (Makishima *et al.*, 2002; Thummel *et al.*, 2001). Our data on the two $1,25(OH)_2D_3$-responsive genes, the D_3 24-hydroxylase and *CYP3A4*, also show as a positive correlation in the induction profile of the two genes. This observation, therefore, suggest that the response of the two genes to calcitriol harbours common elements and that the non-responsive specimen may have exhibited an intrinsic signaling defect to $1,25(OH)_2D_3$ despite appropriate VDR_n mRNA abundance. Moreover, the specimen was also shown, contrary to others, to exhibit no down-regulation in the 1α-hydroxylase mRNA but an up-regulation following exposure to $1,25(OH)_2D_3$. The reason for these differences in response to $1,25(OH)_2D_3$ in this specimen in not clear. A genetic defect in VDR_n responsiveness, the mother's nutritional status (including the D_3 and calcium status), or her exposure to specific nutrients or drugs may be evoked. Indeed, women have been reported to consume an average of 10.3 different drugs during pregnancy and several of these xenobiotics are detectable in the infant's serum at birth (Cresteil, 2001). Moreover, there is a well documented inter-individual variability in cytochrome P-450 responses which may be attributed to genetic factors (polymorphysim, mutations), environmental factors, drug exposure, nutritional status, age, *etc.* In fact, 90% of the inter-individual variability in CYP3A4 activity are presumed to be genetically

determined (Ozdemir *et al.*, 2000).

A response to $1,25(OH)_2D_3$ was also observed for both the 25-hydroxylase and the 1α-hydroxylase with a $1,25(OH)_2D_3$-mediated decrease in the level of expression of both genes. Indeed, the 1α-hydroxylase was shown to rapidly decrease with an observed significant down-regulation at the 24h time-point while a more progressive decrease was observed in the 25-hydroxylase mRNA levels to reach a significant down-regulation at the 48h time-point. The latter observation is in agreement with a previous study showing that in rat intestine both the 25-hydroxylase mRNA and protein levels are decreased by $1,25(OH)_2D_3$ to a level similar to that observed for the D_3 25-hydroxylase mRNA observed in the present study (Theodoropoulos *et al.*, 2001). In the rat intestine, the mechanisms involve both a decrease in mRNA half-life and in the 25-hydroxylase transcription rate which translated in a significant decrease in the activity of the enzyme (Theodoropoulos *et al.*, 2001). Our data thus suggest that in the human intestine, regulation of *CYP27A* is similar to that observed in the young adult rat and that exposure to the D_3 hormone contributes to decrease the abundance of the D_3 25-hydroxylase gene transcript. The down-regulation of the human 1α-hydroxyase gene by $1,25(OH)_2D_3$ is also concordant with observations showing that in murine kidney, the transcription rate of *CYP27B1* is significantly decreased by $1,25(OH)_2D_3$ (Murayama *et al.*, 1999).

Our studies also clearly show the presence of the three key D_3 hydroxylase gene transcripts in freshly harvested fetal intestines as well as in fetal intestines under organotypic culture conditions known to promote differentiation of the organ to exhibit characteristics similar to the normal post-natal intestine (Ménard, 1989). The presence of the three hydroxylase transcripts in the human intestine suggests that the latter is most likely able to produce all the main D_3 metabolites ($25(OH)D_3$, $1,25(OH)_2D_3$,

$24,25(OH)_2D_3$, $1,24,25(OH)_3D_3$) for auto/paracrine purposes to promote either intestinal cell proliferation or differentiation as already shown by Ménard *et al.* (Arsenault & Ménard, 1987; Ménard *et al.*, 1995). Our studies thus clearly illustrate that the human intestine has the potential to fully respond, in a VDR_n-responsive manner, to endogenous or exogenous stimuli as observed with the clear induction of *CYP24* and *CYP3A4*, two major detoxifying cytochrome P-450s. The local association of $1,25(OH)_2D_3$ and the VDR_n in the human intestine also points out that the latter would also be able to respond to intestinal toxins such as lithocholic acid (a suspected intestinal carcinogen) which has recently been shown to induce cytochrome P-450 3A in a VDR_n-dependent manner in an experimental murine model (Makishima *et al.*, 2002). These observations offer a plausible mechanism of action to already known associations, as illustrated by epidemiological and experimental studies, between the incidence of colon cancer or the susceptibility to intestinal DNA damage and vitamin D intake/ultraviolet exposure or the VDR_n genotype (Pence & Buddingh, 1988; Martinez et al 1996; Garland *et al.*, 1999; Kallay *et al.,* 2001). Our data, thus, clearly illustrate the potential importance of the D_3 auto/paracrine system as a key element in the maintenance of the intestinal mucosa integrity in both the basal state and in response to damage-inducing agents.

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TABLE 1

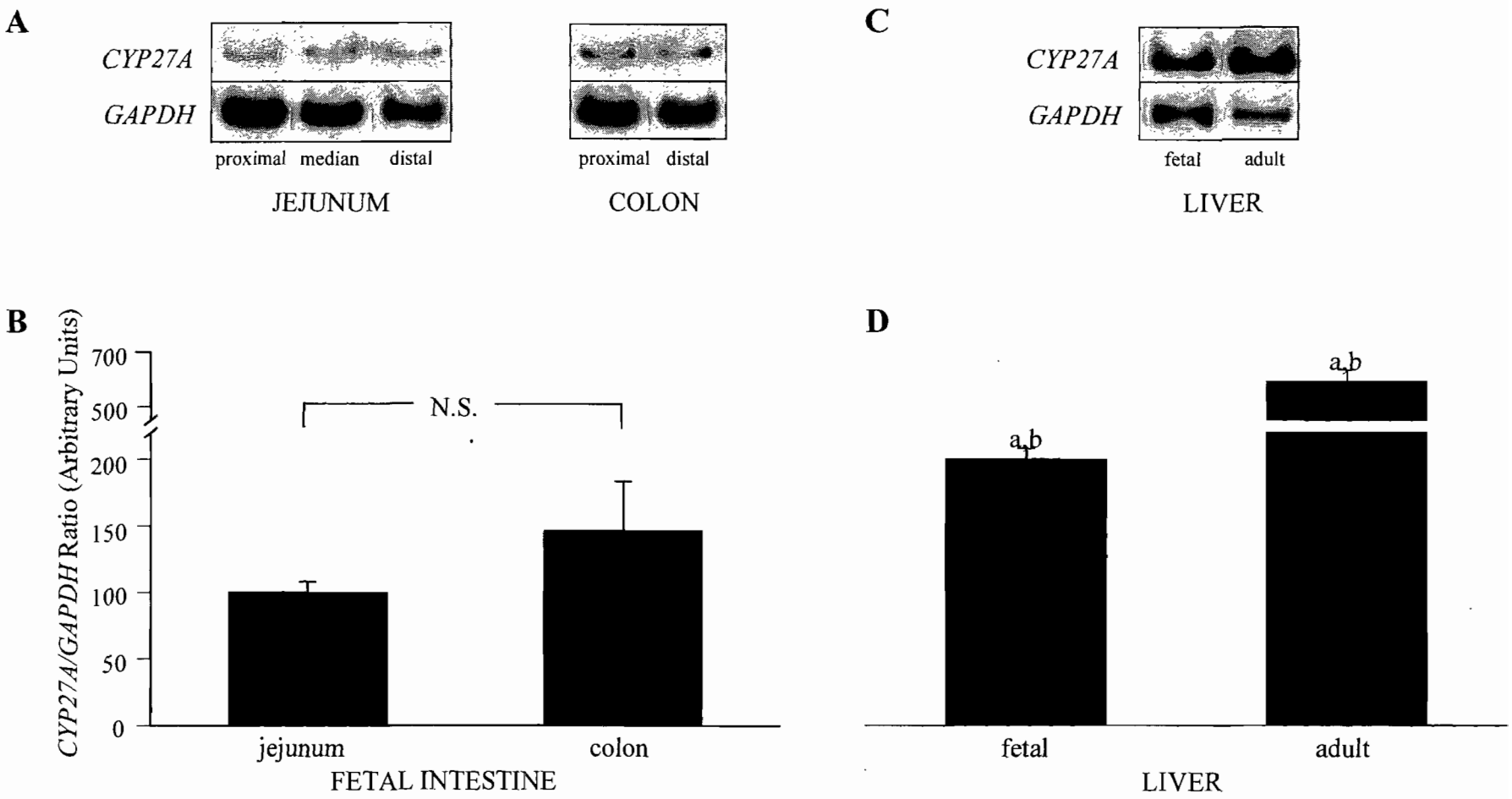
Description of sequence primers:

Primers	Sequences	Base Numbers
1α-Ohase	5' ACGCTGTTGACCATGGC3'	706-722
	5'GTGACACAGAGTGACCAGCATAT3'	1248-1226
24-Ohase	5'GATTCCTTTATGGCATTAGGG3'	2542-2562
	5'AAACTTTGAAACATGCCCTG3'	2752-2733
25-Ohase	5'CAACGGAGCTTAGAGGAGATTC3'	181-202
	5'CCTCATTGAAAGCATCCGTATA3'	571-550
VDR	5'CAGCGGCCAGCACCTCCCTGC3'	99-117
	5'CTGTCCTTCAAGGCCTCTTCC3'	442-426
CYP3A4	5'CCTACATATACACACCCTTTGGAGT3'	1392-1412
	5'AGCTCAATGCATGTACAGAATCCCCGGTTA3'	1770-1741
GAPDH	5'CCCTTCATTGACCTCAACTACATGGT3'	208-233
	5'GAGGGGCCATCCACAGTCTTCTG3'	677-655

LEGEND TO FIGURE 1

Steady state expression of the gene encoding the D_3 25-hydroxylase *CYP27A* in the human fetal intestine. A. Representative *CYP27A* expression profile in the proximal, median and distal jejunum, and proximal and distal colon of fetuses 15 to 20 weeks of gestational age. B. Mean *CYP27A* mRNA levels observed in the fetal jejunum (averaged values of proximal, median and distal) and fetal colon (averaged values of proximal and distal) (n = 5 subjects). C. Representative *CYP27A* expression profile in fetal and adult liver. D. Mean *CYP27A* levels observed in the fetal and adult liver. Data in B and D are presented as mean ± S.E.M. 25 PCR cycles were used and found to be in the linear portion of the *CYP27A* cDNA amplicon generated (20, 25, and 30 PCR cycles, n = 3, r^2 = 0.96, $p<0.001$). 20 PCR cycles were used and found to be in the linear portion of the *GADPH* cDNA amplicon generated (15, 20, and 25 PCR cycles, n = 3, r^2 = 0.97, $p<0.0001$). Statistically significant differences between the group mean *CYP27A* mRNA levels observed in fetal jejunum and fetal colon as well as between the fetal jejunum or the fetal colon and the fetal or adult liver were analyzed by the Student's "t" test; *a* represents the level of significance of the differences between the fetal jejunum and the fetal liver $p<0.0004$, or the adult liver, $p<0.0001$; *b* represents the difference between the fetal colon and the fetal liver, N.S., or the adult liver, $p<0.0001$.

Figure 1


A
CYP27A
GAPDH
proximal median distal
JEJUNUM
proximal distal
COLON
C
CYP27A
GAPDH
fetal adult
LIVER
B
CYP27A/GAPDH Ratio (Arbitrary Units)
700
500
200
150
100
50
0
N.S.
jejunum
colon
FETAL INTESTINE
D
a,b
a,b
fetal
adult
LIVER

LEGEND TO FIGURE 2

Effect of $1,25(OH)_2D_3$ exposure on the expression of the gene encoding the D_3 25-hydroxylase *CYP27A* in the human fetal jejunum. Specimens were incubated in the presence of 10^{-7}M $1,25(OH)_2D_3$ for a period of 24h (n = 5 subjects) or 48h (n = 4 subjects). Data are presented as % (mean ± S.E.M.) differences over the value obtained in paired fetal intestinal specimens obtained from the same subject incubated in the absence of $1,25(OH)_2D_3$. Statistically significant differences between the group mean mRNA levels observed in the presence or absence of $1,25(OH)_2D_3$ were analyzed by the paired Student's "t" test, *$p<0.003$.

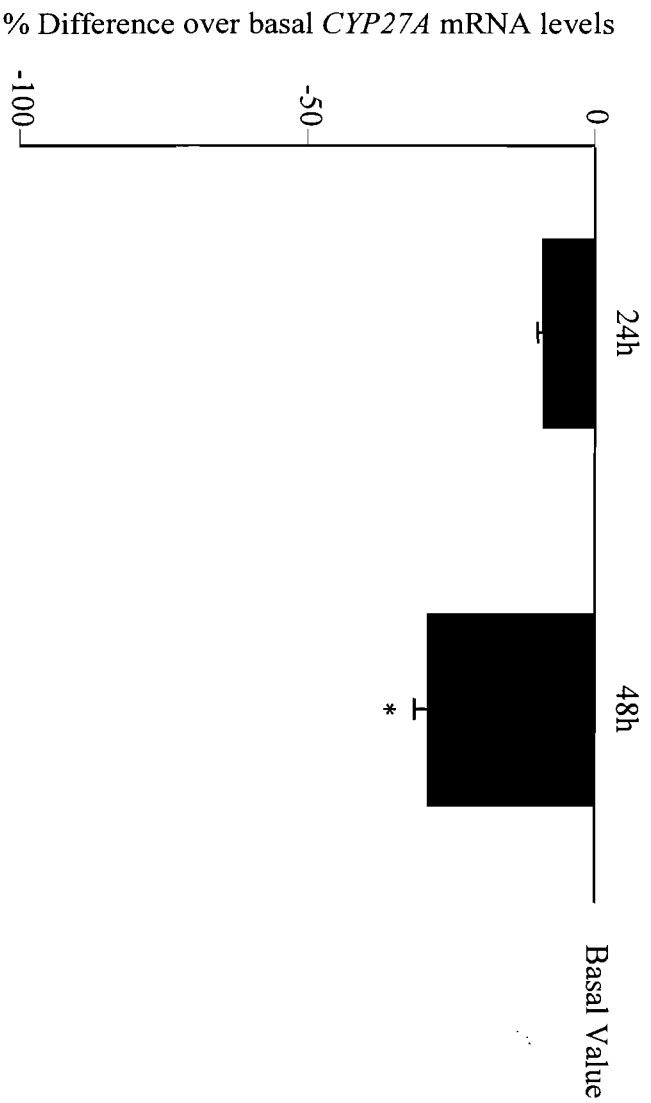
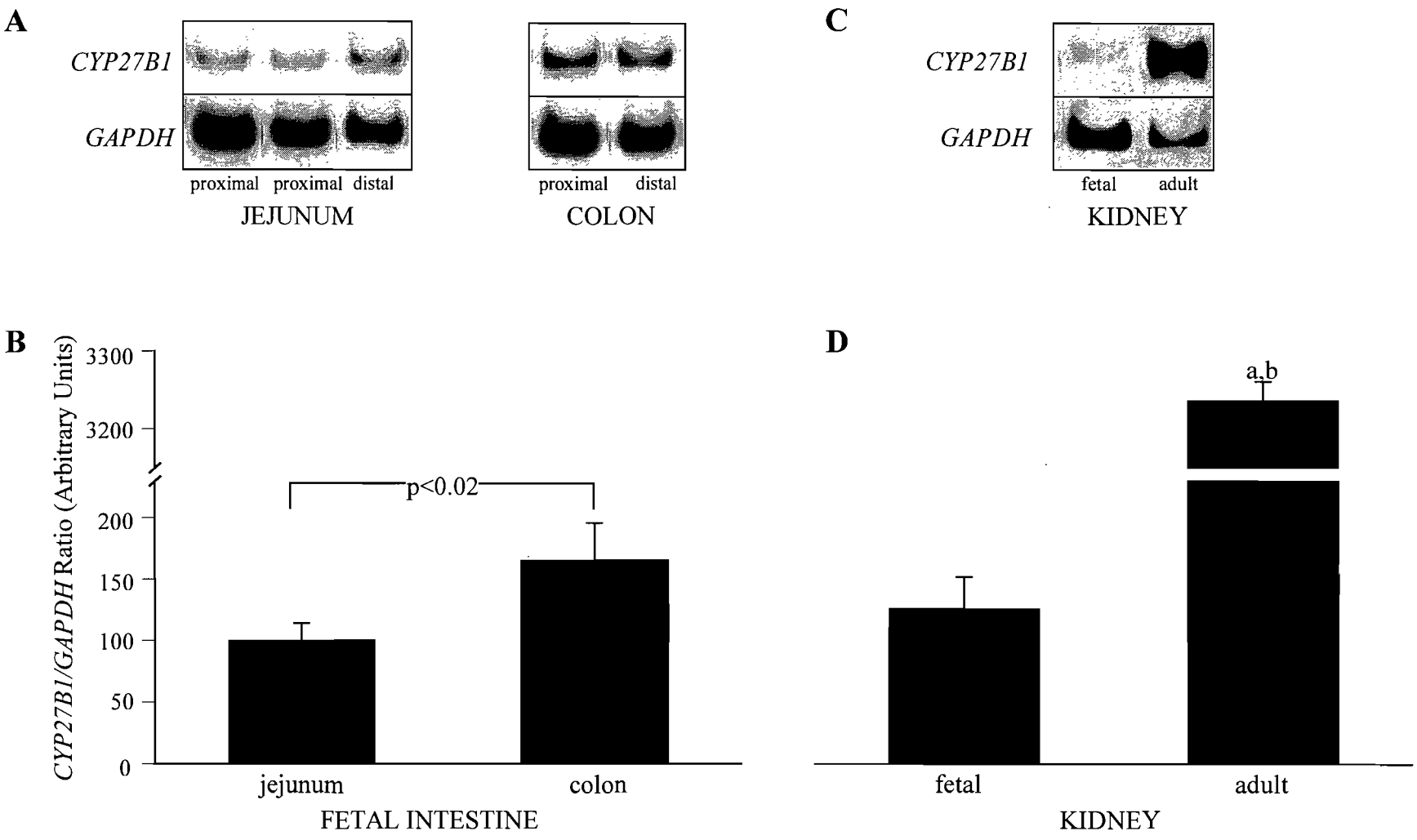

% Difference over basal CYP27A mRNA levels
-100
-50
0
24h
48h
*
1,25(OH)2D3 EXPOSURE
Basal Value

Figure 2

LEGEND TO FIGURE 3

Steady state expression of the gene encoding the D_3 1α-hydroxylase *CYP27B1* in the human fetal intestine. A. Representative *CYP27B1* expression profile in the proximal, median and distal jejunum, and proximal and distal colon of fetuses 15 to 20 weeks of gestational age. B. Mean *CYP27B1* mRNA levels observed in the fetal jejunum (averaged values of proximal, median and distal) and fetal colon (averaged values of proximal and distal) (n = 5 subjects) and fetal colon (averaged values of proximal and distal) (n = 4 subjects). C. Representative *CYP27B1* expression profile in the fetal and adult kidney. D. Mean *CYP27B1* levels observed in the fetal and adult kidney. Data in B and D are presented as mean ± S.E.M. 30 PCR cycles were used and found to be in the linear portion of the *CYP27B1* cDNA amplicon generated (25, 30, and 35 PCR cycles, n = 3, r^2 = 0.67, $p<0.004$). Statistically significant differences between the group mean *CYP27B1* mRNA levels observed in fetal jejunum and fetal colon as well as between the fetal or the fetal colon and the fetal or adult kidney were analyzed by the Student's "t" test; *a* represents the level of significance of the differences between the fetal jejunum and the fetal kidney, N.S., or the adult kidney, $p<0.0001$; *b* represents the difference between the fetal colon and the fetal kidney, N.S., or the adult kidney, $p<0.0001$.

Figure 3


A
CYP27B1
GAPDH
proximal proximal distal
JEJUNUM
proximal distal
COLON
C
CYP27B1
GAPDH
fetal adult
KIDNEY
B
CYP27B1/GAPDH Ratio (Arbitrary Units)
3300
3200
200
150
100
50
0
p<0.02
jejunum
colon
FETAL INTESTINE
D
a,b
fetal
adult
KIDNEY

LEGEND TO FIGURE 4

Effect of $1,25(OH)_2D_3$ exposure on the expression of the gene encoding the D_3 1α-hydroxylase *CYP27B1* in the human fetal jejunum. Specimens were incubated in the presence of 10^{-7}M $1,25(OH)_2D_3$ for a period of 24h (n = 5) or 48h (n = 4). Data are presented as % (mean ± S.E.M.) differences over the value obtained in paired fetal intestinal specimens obtained from the same subject incubated in the absence of $1,25(OH)_2D_3$. Statistically significant differences between the group mean mRNA levels observed in the presence or absence of $1,25(OH)_2D_3$ were analyzed by the paired Student's "t" test, *$p<0.05$.

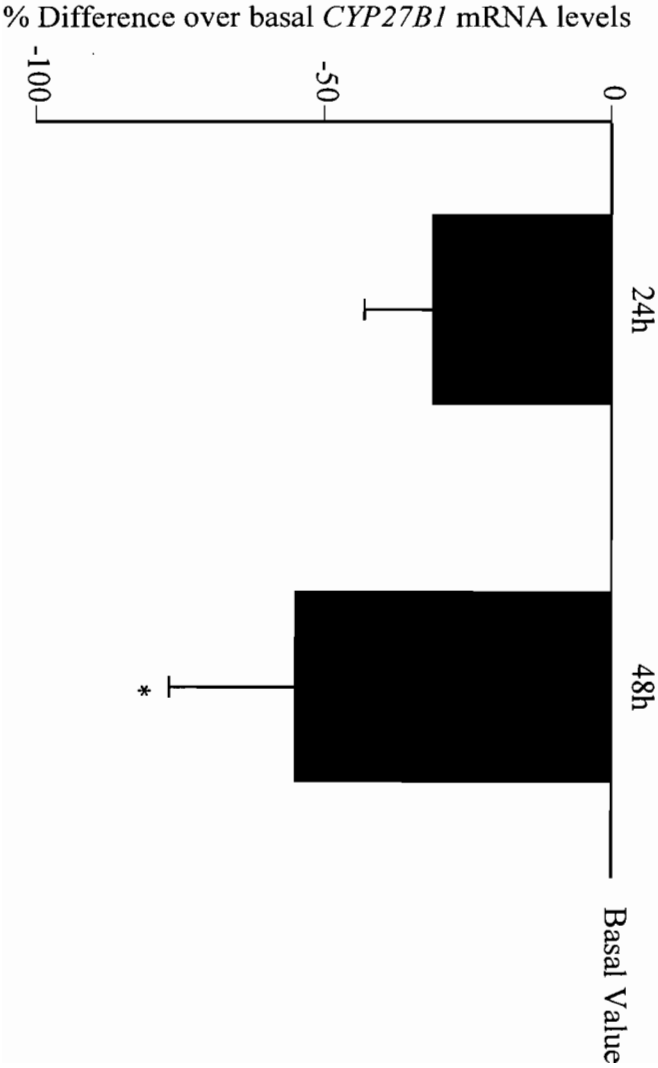
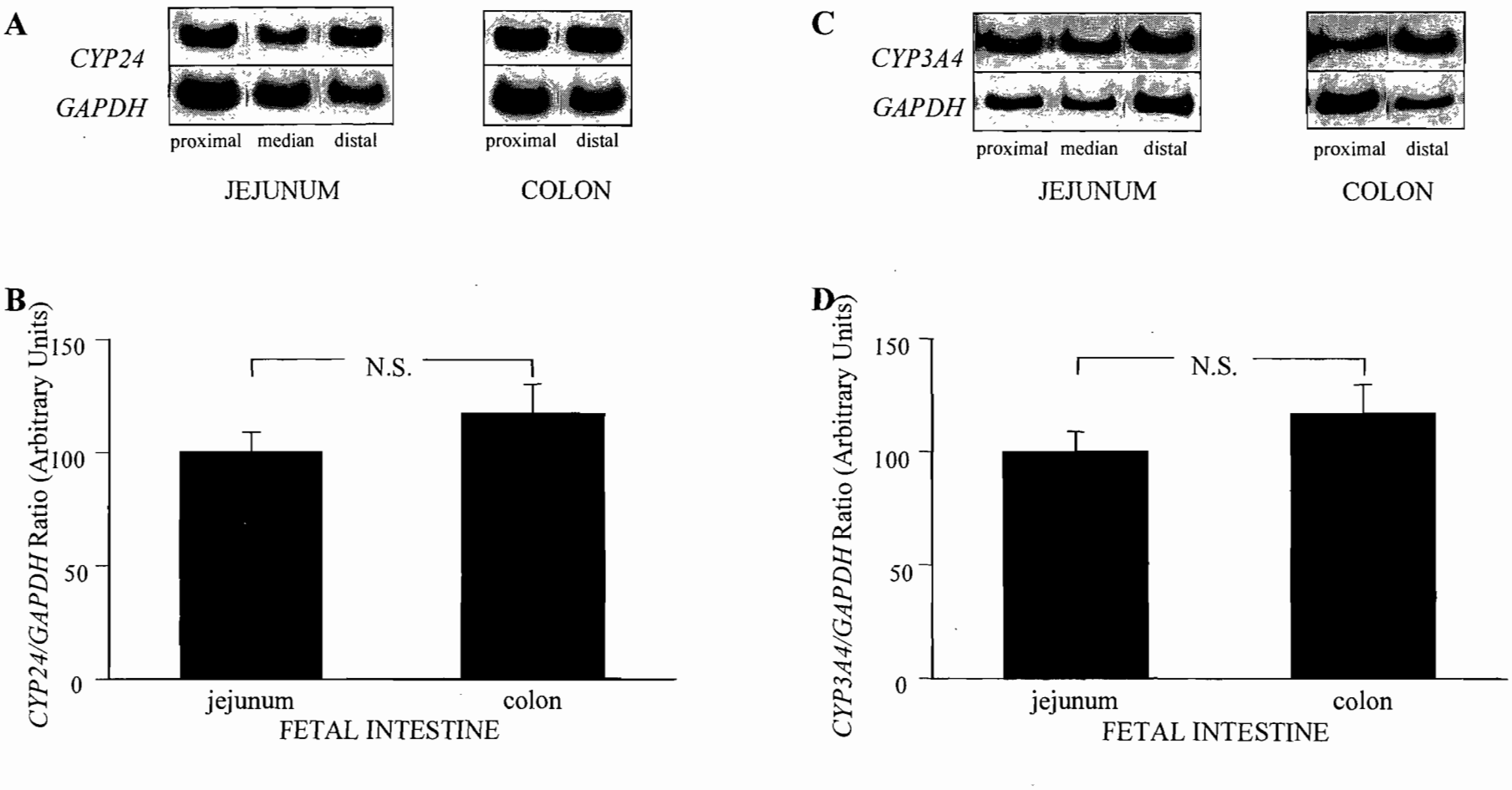

% Difference over basal *CYP27B1* mRNA levels
-100
-50
0
24h
48h
Basal Value
1,25(OH)2D3 EXPOSURE
*

Figure 4

LEGEND TO FIGURE 5

Steady state expression of the genes encoding the D_3 24-hydroxylase *CYP24* and *CYP3A4* in the human fetal intestine. A. Representative *CYP24* expression profile in the fetal proximal, median and distal jejunum, and fetal proximal and distal colon. B. Mean ± S.E.M. *CYP24* mRNA levels observed in the fetal jejunum (averaged values of proximal, median and distal) (n = 5 specimens) and fetal colon (averaged values of proximal and distal) (n = 4 specimens). 30 PCR cycles were used and found to be in the linear portion of the *CYP24* cDNA amplicon generated (25, 30, and 35 PCR cycles, n = 3, $r^2 = 0.83$, $p<0.001$). C. Representative *CYP3A4* expression profile in the proximal, median and distal jejunum and the proximal and distal colon. D. Mean ± S.E.M. *CYP3A4* mRNA levels observed in the fetal jejunum (n = 5 specimens) and fetal colon (proximal and distal) (n = 2 specimens). 30 PCR cycles were used and found to be in the linear portion of the *CYP3A4* cDNA amplicon generated (25, 30, and 35 PCR cycles, n = 3, $r^2 = 0.80$, $p<0.0007$). Statistically significant differences between the group mean mRNA levels were analyzed by the Student's "t" test.

Figure 5


A
CYP24
GAPDH
proximal median distal
JEJUNUM
proximal distal
COLON
C
CYP3A4
GAPDH
proximal median distal
JEJUNUM
proximal distal
COLON
B
CYP24/GAPDH Ratio (Arbitrary Units)
150
100
50
0
N.S.
jejunum
colon
FETAL INTESTINE
D
CYP3A4/GAPDH Ratio (Arbitrary Units)
150
100
50
0
N.S.
jejunum
colon
FETAL INTESTINE

LEGEND TO FIGURE 6

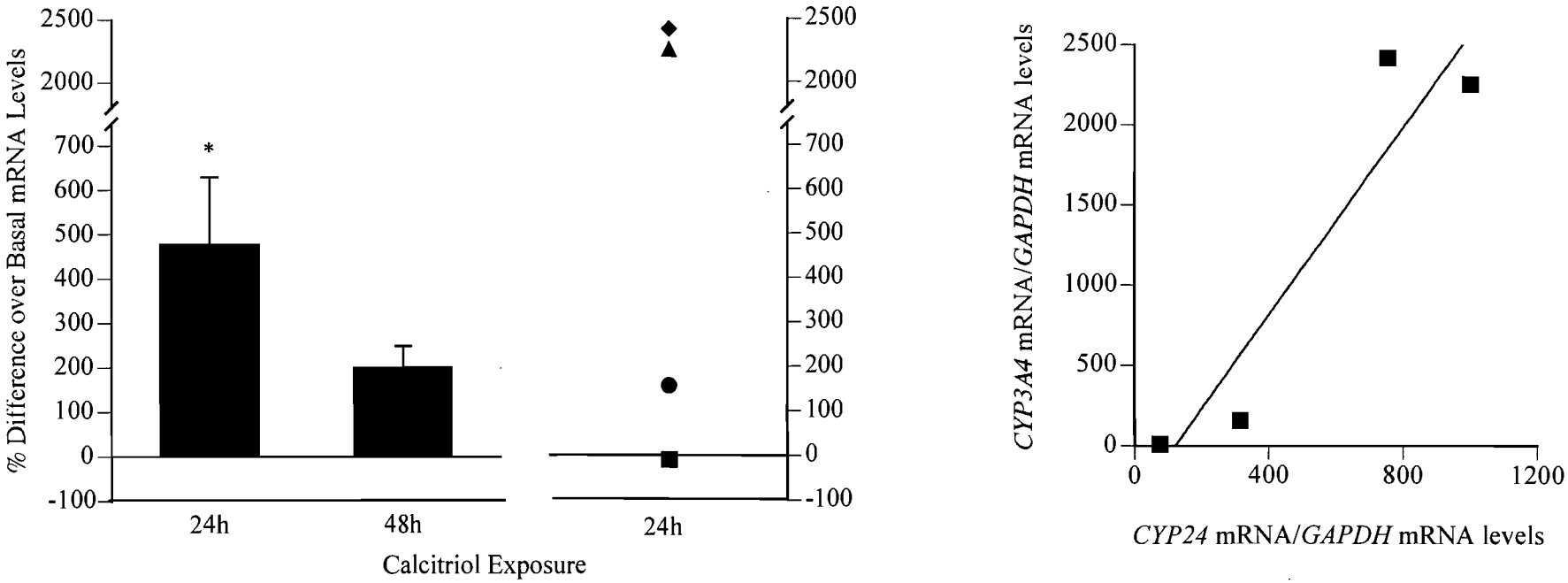
Effect of $1,25(OH)_2D_3$ exposure on the expression of the genes encoding the D_3 24-hydroxylase *CYP24* (A) and *CYP3A4* (B) in the human fetal jejunum. Specimens were incubated in the presence of 10^{-7}M $1,25(OH)_2D_3$ for a period of 24h or 48h. Data for are presented as % differences over the value obtained in paired fetal intestinal specimens obtained from the same subject incubated in the absence $1,25(OH)_2D_3$. Statistically significant differences between group *CYP24* mean mRNA levels observed in the presence or absence of $1,25(OH)_2D_3$ were analyzed by the paired Student's "t" test, *CYP24*: n = 6 at the 24h time-point and (n = 4) at the 48h time point, *$p<0.03$. *CYP3A4*: n = 4. C. Pearson's correlation coefficient between *CYP24* and *CYP3A4* mRNA levels following 24h $1,25(OH)_2D_3$ exposure. n = 4, $r^2 = 0.80$, $p<0.03$.

Figure 6

A. *CYP24*

B. *CYP3A4*

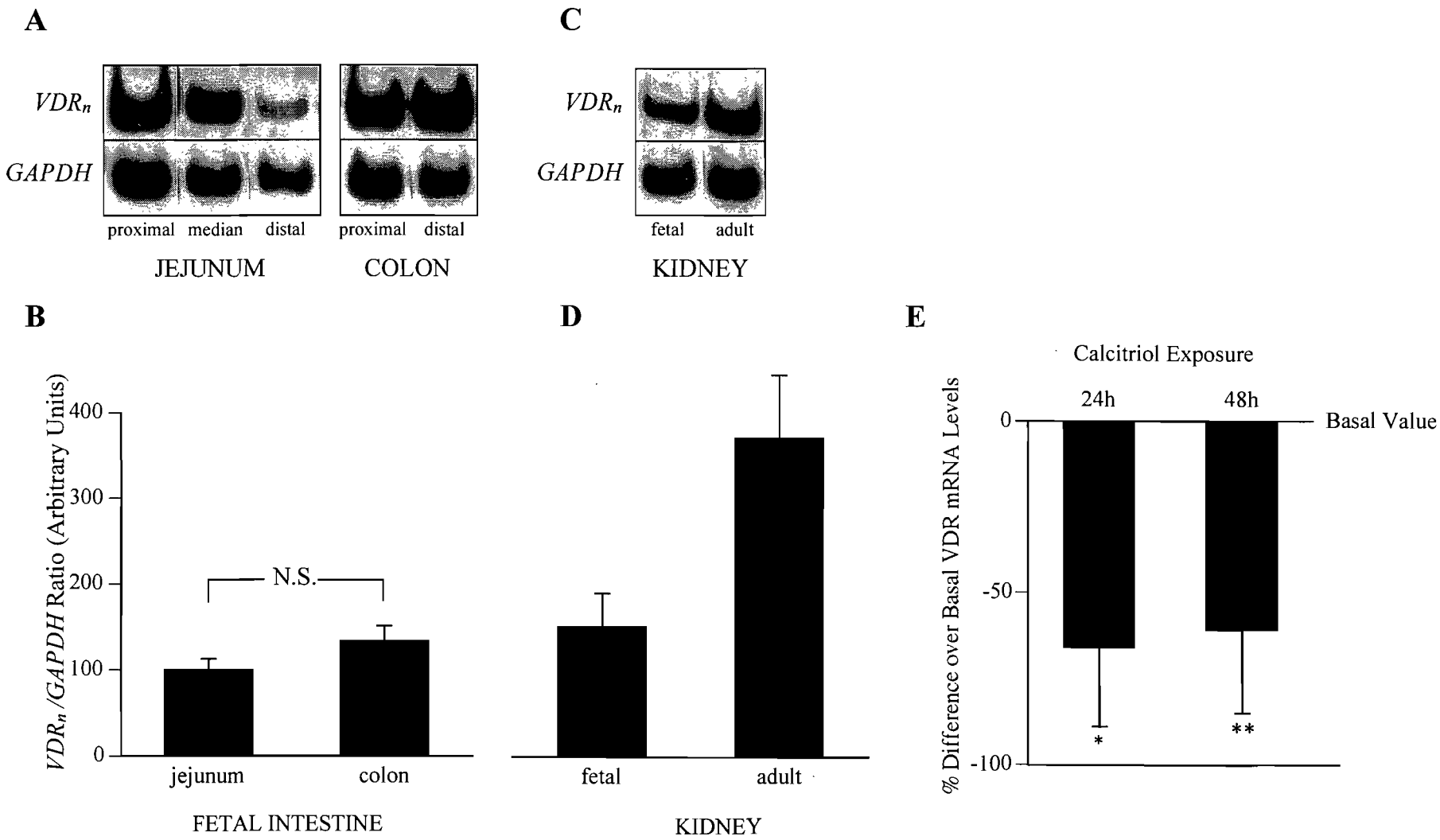
C.



LEGEND TO FIGURE 7

Steady state expression of the gene encoding the nuclear vitamin D receptor VDR_n in the human fetal intestine. A. Representative VDR_n expression profile in the proximal, median and distal jejunum, and proximal and distal colon of fetuses 15 to 20 weeks of gestational age. B. Mean ± S.E.M. *VDR* mRNA levels observed in the fetal jejunum (averaged values of proximal, median and distal) (n = 5 subjects) and fetal colon (averaged values of proximal and distal) (n = 3 subjects). Statistically significant differences between the group mean mRNA levels were analyzed by the Student's "t" test. C. Representative VDR_n expression profile in fetal and adult kidney. D. Mean VDR_n levels observed in the fetal and adult kidney. E. Effect of $1,25(OH)_2D_3$ exposure on the expression of the genes encoding VDR_n in the human fetal jejunum. Specimens were incubated in the presence of 10^{-7}M $1,25(OH)_2D_3$ for a period of 24h (n = 4) or 48h (n = 4). Data are presented as % (mean ± S.E.M.) differences over the value obtained in paired fetal intestinal specimens obtained from the same subject incubated in the absence of $1,25(OH)_2D_3$. 27 PCR cycles were used and found to be in the linear portion of the VDR_n cDNA amplicon generated (22, 27 and 32 PCR cycles, n = 3, $r^2 = 0.91$, $p<0.0001$). Statistically significant differences between the group mean mRNA levels observed in the presence or absence of $1,25(OH)_2D_3$ were analyzed by the paired Student's "t"; * $p<0.005$, ** $p<0.03$.

Figure 7


A
VDRn
GAPDH
proximal median distal
proximal distal
JEJUNUM
COLON
C
VDRn
GAPDH
fetal adult
KIDNEY
B
VDRn /GAPDH Ratio (Arbitrary Units)
400
300
200
100
0
N.S.
jejunum
colon
FETAL INTESTINE
D
fetal
adult
KIDNEY
E
Calcitriol Exposure
24h
48h
0
-50
-100
Basal Value
% Difference over Basal VDR mRNA Levels
*
**

3.2.3 Additional Results

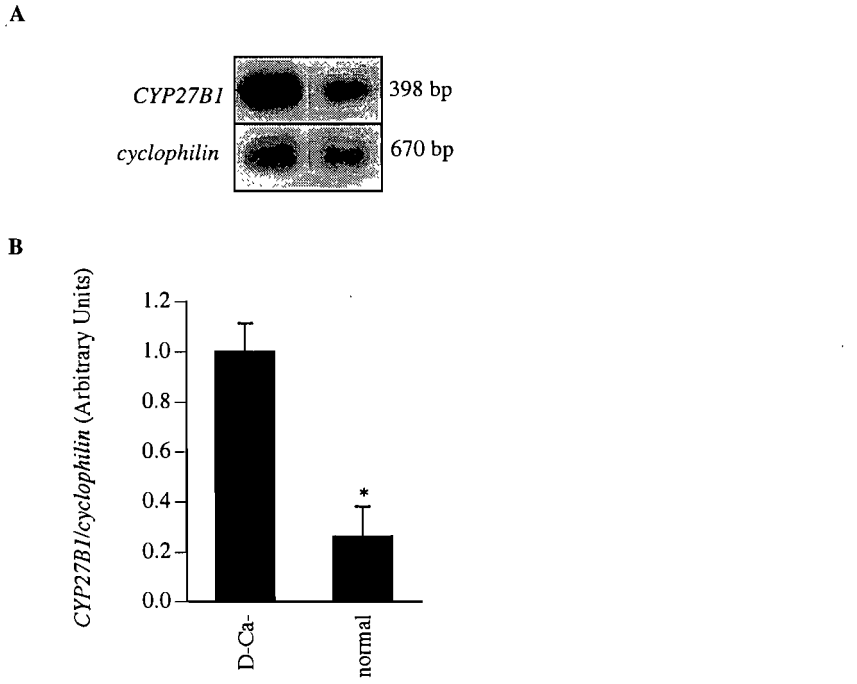


Figure 3.1: Expression of *CYP27B1* in normal and D-Ca- rats. *CYP27B1* mRNAs were reversely transcribed into cDNAs and amplified by PCR (25 cycles for *CYP27B1* and 20 cycles for *cyclophilin*, control gene), using specific probes (see method details in Article 4). cDNA formed was loaded on a 1.2% agarose gel and transferred onto a Southern blot which was then hybridized with the appropriate probe. Band intensity was then analysed by densitometric analysis. A. Representative southern blot of amplified *CYP27B1* cDNA. B. Relative intensity of *CYP27B1* in normal and D-Ca- rats. Results are presented as mean ± S.E.M. Statistically significant differences were evaluated by the Student's t test, *$p<0.05$; normal, n=6; D-Ca-, n=11.

Results: *CYP27B1* mRNA levels were 4-fold less abundant in rats fed a normal diet compared to those subjected to a D-depleted diet ($p<0.05$).

3.3 CONCLUSIONS

These experiments show that the intestine harbours all three D_3 hydroxylases. Intestinal *CYP27A* mRNA steady state levels were three times less abundantly expressed than in liver and was downregulated by all major D_3 metabolites, D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$, by an effect on the transcription rate. The strong *CYP24* induction following $1,25(OH)_2D_3$, administration, underlies the importance of this cytochrome in D_3 metabolite catabolism. Furthermore, *CYP27B1* proved to be sensitive to the D status.

All hydroxylases, *CYP3A4* as well as the *VDR* were expressed along the human fetal intestine and colon. However, only *CYP27B1* levels were significantly upregulated in the latter part. The sensitivity of the hydroxylases, *CYP3A4* and the *VDR* to $1,25(OH)_2D_3$ administration establish the intestine as an organ capable of autoregulatory control through hydroxylase modulation by an autocrine/ paracrine mechanism of regulation.

CHAPTER 4: OVERALL DISCUSSION AND CONCLUSIONS

In the present work, we have examined hepatic and intestinal *CYP27A* expression in relation to D homeostasis in both the rat and the human. The first part illustrates the effect of the D hormonal and nutritional status on hepatic *CYP27A* in the liver, the primary and major organ in D_3 hydroxylation (164). Intestinal *CYP27A* regulation was then evaluated in relation to the other hydroxylases present in this organ, *CYP27B1*, *CYP24* and *CYP3A4*. Our findings illustrate for the first time the sensitivity of *CYP27A*, to D homeostasis, and therefore open further opportunities for investigation and debate into the role and regulation of this cytochrome in relation to D metabolism.

Little is known regarding the regulation of 25(OH)D production. Indeed, the apparent non regulation in D mitochondrial 25-hydroxylation (21;22) is contradictory to the tight regulatory control mechanisms reported for its hydroxylase counterparts CYP27B1 and CYP24.

The liver and intestine being closely linked by the enterohepatic circulation, which prolongs the presence of D metabolites and increases their exposure to CYP27A, making these tissues highly relevant in their regulation.

Previous *in vivo* studies, based primarily on enzymatic activities, have been inconclusive with respect to the effects of the D status on hepatic 25-hydroxylation and have solely focused on this tissue. Our results clearly show the sensitivity of hepatic CYP27A gene to only $1,25(OH)_2D_3$ administration in rats, and a correlation between circulating 25(OH)D concentrations and *CYP27A* mRNA steady state levels in humans. This correlation implies a link between the nutritional status and the gene levels ($r^2=0.41$, $p<0.05$) .

Interestingly, rat hepatic *CYP27A* mRNA levels proved to be sensitive only to the i.v. administration of hormonal form of the vitamin, $1,25(OH)_2D_3$, and not to D_3 or $25(OH)D_3$. Thus, although a *CYP27A* interspecies similarity in sex regulated mechanisms is observed, the role of the D status is not as clear-cut. As may be encountered in other studies, the regulatory pattern and mechanisms of a rat specific gene cannot be fully extrapolated into its human counterpart, consequently supporting that studies must be fully and thoroughly undertaken to obtain a complete and clear understanding of human gene regulation.

Our findings suggest that different metabolites and/or mechanisms are affecting *CYP27A* gene levels between these two species. It appears that the human *CYP27A* is sensitive to the 25(OH)D concentrations whereas in the rat it is sensitive to only $1,25(OH)_2D_3$ i.p. administration.

We are lead to hypothesise that in the D-Ca- rat model under investigation, some other metabolite(s) produced downstream of $1,25(OH)_2D_3$ may be responsible for *CYP27A* downregulation by a marked effect on the transcription rate, whereas the half-life remains unaffected. Our studies using ketoconazole further support this hypothesis. Ketoconazole administration together with $1,25(OH)_2D_3$ inhibited the *CYP27A* downregulation observed with $1,25(OH)_2D_3$ alone, suggesting further P450 modification is required for an effect on *CYP27A*. Indeed, Harant *et al.* (241) have demonstrated that along the D catabolic pathway, biologic activity of some natural metabolites is retained despite an apparently lower affinity for VDR, that normally regulates VDR responsive genes. However, we cannot confirm that a D_3 metabolite is in fact affecting gene levels. Moreover, the question arises as to the mechanism of transcriptional inhibition and of the presence and functionality of a VDR in the liver. To date, this receptor in the liver has been characterised by certain investigators (190;191), including our group who has identified the VDR by RT-PCR in the nonparenchymal cells and to a lesser extent in the hepatocyte (242). We may also speculate that another type of receptor, abundantly present in the liver, may be involved. No studies have addressed binding of $1,25(OH)_2D_3$ to other types of receptors.

Unfortunately, the lack of information about the precise sequences upstream of the transcriptional initiation sites remain unpublished by the two groups of investigators who have published regulatory studies (52;53). Their hopeful identification in the near future will answer the questions pertaining to potential receptor (i.e. VDR) and transcription factor binding sites.

Alternatively, a bile acid metabolite may be responsible for the *CYP27A* gene regulation observed since $1,25(OH)_2D_3$ undergoes glucuronidation and excretion into bile. This would not be surprising, since CYP27A is indeed an enzyme downregulated by bile acids via a transcriptional mechanism (227). The enzyme also exhibits a higher degree of hydroxylation towards cholesterol than D_3 (227). Interestingly, *CYP7*α, the enzyme involved

in the neutral pathway of bile acid synthesis, was also downregulated by $1,25(OH)_2D_3$ supplementation, further strengthening this hypothesis. D_3 and $25(OH)D_3$ proved to have no effect (results not shown). Therefore, as reported in previous studies on the effect of bile acids in rat livers, *CYP7*α and *CYP27A* mRNA levels were observed to change in the same direction (227;243;244).

Recently, lithocholic acid (LCA) and its major metabolites 3'keto-LCA, glyco-LCA and 6'keto-LCA have been reported to activate VDR and the expression of CYP3A (17). The concentration at which these bile acids activate VDR is below the pharmacologic range that activates the other bile acid receptors such as farnesoid X receptor (FXR) and pregnane X receptor (PXR), thus strengthening the physiological significance of the VDR binding (17). These data suggest that VDR is a more sensitive receptor for bile acids than are FXR and PXR, and particularly for LCA and its major metabolite 3'keto-LCA (17).

The conclusions regarding the biologic relevance and importance of the CYP27A and other potential 25-hydroxylases require additional research. Although mitochondria were previously believed to be the only organelle possessing 25-hydroxylase activity in humans (27;43), a recent paper indicates microsomes also possess this activity (28), confirming an older study (245). Our studies raise the question of the physiologic significance and role of CYP27A in D_3 homeostasis. It has been previously reported that rats fed D deficient diets and supplemented with physiologic amounts of D_2 have $25(OH)D_2$ as their predominant monohydroxylated D_2 metabolite in plasma (62), suggesting that CYP27A is not the physiologic enzyme responsible for 25-hydroxylation of D, since CYP27A is unable to hydroxylate D_2 (47). The question thus arises as to the physiological significance of CYP27A in terms of D homeostasis, since it is primarily considered to be an enzyme involved in bile acid biosynthesis. The lack of development of CTX abnormalities in the CYP27A -/- mouse, presumably due to the role of the microsomal 25-hydroxylase(s) (246), exemplifies the redundancy in 25-hydroxylase activity. Implicating CYP27A in D_3 metabolism is the fact that some patients suffering from CTX have been reported to exhibit abnormal bone metabolism as well as early osteoporosis (69). However mutations affecting CYP27A do not seem to completely abolish the C-25 hydroxylation of D_3 (67;69;71). This observation suggests the presence of an alternative, but as of yet, unidentified D_3 25-

hydroxylase(s) in humans, possibly an equivalent to the pig microsomal D_3 25-hydroxylase (32). The molecular identity is still not known. Upon cloning and identification of this/these hydroxylase(s), it will be of great interest to examine their response in relation to D homeostasis.

Furthermore, our observations indicate that *CYP27A* expression can be significantly upregulated in certain pathological situations such as in hepatic carcinoma and in intrahepatic metastasis of the colon and that the neoplastic tissue could contribute to the circulating 25(OH)D concentration. It is clear that CYP27A also plays a role in disease. Beyond its involvement in CTX, it is involved in the metabolism of oxysterols such as 7-ketocholesterol, which is potentially pro-atherogenic, and is a quantitatively important oxysterol in both atherosclerotic lesions and macrophage foam cells (247). Studies with CYP27A-/- mice have shown increases in triacylglycerol and hepatic synthesis of fatty acids, which is associated with hypertriglyceridemia and hepatomegaly (248). These findings confirm the importance of CYP27A in bile acid synthesis and reveal a function of the enzyme in triacylglycerol metabolism. The presence of CYP27A in a wide variety of tissues, contrary to CYP7α, which is confined exclusively to the liver, suggests a global importance of CYP27A in the maintenance of cellular integrity. It will be of interest to determine (knowing levels may be upregulated in certain pathologies) whether the hepatic signal distribution may vary in the state of malignancy since in the normal state it is confined to the PV region, as evidenced by *in situ* hybridization in humans. To our knowledge, this is the first time *CYP27A* upregulation is linked to non-atherogenic type pathologies (hepatic carcinoma and intrahepatic colon metastasis), indicating a potentially more widespread involvement in other disease states, and the possible involvement of D homeostasis.

Although, CYP27A is most abundantly expressed in the liver, it is also present in many other tissues including kidney, adrenals, testes and the intestine (234). In the intestine, its mRNA steady state expression is three times less than in the liver. Our study examines for the first time the regulation of this gene in the intestine.

In addition to the presence and regulation of both *CYP24* and *CYP27A* by the D_3 status, we were able to characterise the presence of *CYP27B1* in the rat intestine. Expression levels in the D-Ca- rat proved to be significantly higher than those observed in

the normal intestine. The increase in gene expression levels may be necessary in the D-Ca-rat in order to maximize and potentialize the production of the $1,25(OH)_2D_3$ for local and/or systemic actions. This hypothesis remains to be investigated.

The intestine has long been regarded as a classic D response tissue. D is well known to have profound effects upon the small intestine through upregulation of calbindin 9K, the cytosolic calcium binding protein (249), as well as calcium pumps situated on the basolateral membrane of small intestinal epithelial cells (250). The cellular effects are believed to greatly enhance the transport of calcium from the lumen of the small intestine and colon into the bloodstream (251;252). Furthermore, until recently, it has been believed that virtually all circulating $1,25(OH)_2D_3$ is formed in the kidney from $25(OH)D_3$, based upon the observation that nephrectomy almost completely depletes circulating $1,25(OH)_2D_3$(253). The present work thus changes the status of this organ from a simple D_3 target organ, to one potentially autoregulatory in its own actions through hydroxylase modulation. Recent findings in the field of colon carcinogenesis suggest the possibility of a local effect of $1,25(OH)_2D_3$ through the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ (17).

Contrary to intestinal *CYP27A* and *CYP27B1*, regulatory control of *CYP24* has been well documented. As opposed to the on/off switch observed with the *CYP24* intestinal regulation, *CYP27A* levels in both the intestine and in the liver were not completely decreased despite the high pharmacological doses of $1,25(OH)_2D_3$ administered. These findings suggest the potential need of basal expression levels, evoking a more widespread role than solely in D metabolism, and most probably in bile acid metabolism. *CYP27A* has even been proposed as a potential renal D_3 1α-hydroxylase (254).

Thus, the proven regulatory control following D supplementation of D-Ca- rats, with all three hydroxylases affected, suggests that the intestine is a tissue capable of autoregulatory control in terms of D_3 homeostasis. It remains to be investigated whether hydroxylase changes are related to the control of calcium and/or phosphorus homeostasis and/or local hormonal effects on the tissue itself. Indeed, $1,25(OH)_2D_3$ affects intestinal physiology. Furthermore, the presence and regulatory control by D_3 may also open the door to potential treatment in gastrointestinal disease, through the use of D_3 metabolites. Low levels of circulating D_3 have been proposed as a risk factor for distal colorectal adenomas

(255). The use of D_3 metabolites as therapeutic agents is already the subject of many studies and has provided some promising results in therapy (256;257). The development of nontoxic analogs and the understanding of their molecular mechanism of action will be of significant importance in the prevention and treatment of cancer by D_3.

Furthermore, we studied the effect of $1,25(OH)_2D_3$ hormonal action on D_3 hydroxylase regulation. The effect of $1,25(OH)_2D_3$ was investigated in an organotypic human intestinal culture model where $1,25(OH)_2D_3$ functions as a hormonal agent with respect to cellular proliferation and differentiation (233). The intestinal fetal model under investigation, although corresponding to the mid-gestational period in development, is fully developed and is similar to that of the adult human intestine (205).

Basal expression levels of the D_3 hydroxylases *CYP27A* and *CYP24* did not vary along the intestine and colon, unlike *CYP27B1* which was significantly higher in the colon. Overall, it is believed cytochrome P450 activity tends to decrease along the intestine (258), however an exception (2-aminofluorene acetylase activity) has been reported (259). The expression pattern of specific P450s has not been previously addressed. The levels obtained in our study may however be specifically characteristic of the fetal stage.

Our results show that all three D_3 hydroxylases are regulated by $1,25(OH)_2D_3$ in the human intestinal explant cultures in our study. *CYP27A* and *CYP27B1* were significantly downregulated after 48h of culture, whereas *CYP24* was upregulated after 24h. An interindividual variability in response was observed between the samples in the case of *CYP24*, with some responding much more than others, and one sample not responding at all. Interestingly, such a type of P450 variability has also been observed in cultured lung explants (260). These data may be explained by polymorphisms in VDR as well as in the specific hydroxylases, and illustrate the non-uniform response that may be obtained when studying human samples. Unfortunately, due to ethical reasons, we were unable to obtain information on the D_3 status and overall health of the mother. The access to such data would have permitted a more analytical understanding and strengthened the study.

Furthermore, $1,25(OH)_2D_3$ highly induced *CYP3A4*, a P450 involved in xenobiotic metabolism. Several endogenous and exogenous ligands acting through the PXR and SXR induce CYP3A4. CYP3A4 is also inducible by a non-classical mechanism involving binding

of the heterodimer VDR/RXR, not to a VDRE, but to PXRE in PXR independent fashion (261). These findings suggest a more widespread role of D_3 in the regulation of cytochromes also involved in detoxification processes, in addition to the hydroxylases directly involved in its own anabolism and catabolism.

Overall, our findings directly demonstrate the sensitivity of hepatic *CYP27A* expression levels to D_3 deficiency, pathologies and repletion with $1,25(OH)_2D_3$. Intestinal *CYP27B1* was also observed to be sensitive to the D_3 deficient state and to D_3, 25(OH) D_3 and $1,25(OH)_2D_3$ repletion. The homeostatic changes related to the D status that occur following an alteration in the D_3 endocrine status affects the cytochromes involved not only in the vitamin's own anabolism/catabolism, but also others such as CYP3A4. These findings are important, keeping in mind also that the D_3 hydroxylases may be involved in other regulatory mechanisms, as is the case in bile acid regulation for CYP27A. Alterations in the D_3 endocrine system remain an ever-present concern in today's society, with the high prevalence of D_3 depletion in the elderly, and the increased risk of developing D_3 deficiency in healthy individuals. Our studies detail how *CYP27A*, but also other cytochromes involved in drug metabolism may be affected.

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